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ORIGINAL ARTICLE

MGN-3 arabinoxylan rice bran modulates innate immunity in multiple myeloma patients

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Abstract Dendritic cells (DCs) and natural killer (NK) cells are central components of innate immunity for controlling tumor growth. The therapeutic effects of certain anti-myeloma drugs are partially mediated by targeting the innate immune response. In addition, novel types of natural compounds have been developed that efficiently modulate the activity of both the cellular and humoral compartments of immunity. MGN-3 is known as an activator of natural killer cells, inducer of apoptosis and cytokine production, and modulator of dendritic cell maturation and differentiation in vitro. We have performed a randomized, placebo-controlled study to examine the effects of MGN-3 on innate immune system parameters in 48 multiple myeloma patients. We performed immunophenotypic analysis of peripheral blood samples, determined NK cell activity, and assessed the cytokine profiles of plasma before and during 3 months of treatment. The results demonstrate a clear increase in NK activity in MGN-3-treated patients compared to the placebo group, an increased level of myeloid DCs in peripheral blood, and augmented concentrations of T helper cell type 1-related cytokines. The present study suggests that MGN-3 may represent an immunologically relevant product for activating innate immunity in multiple myeloma patients and warrants further testing to demonstrate clinical efficacy.

Keywords Innate immunity · Dendritic cells · Natural killer cells · Cytokines · MGN-3 · Multiple myeloma

Introduction

Different cell types within the bone marrow (BM), including cells of the immune system, mesenchymal stem cells, and BM stromal cells, can contribute to the development of the disease multiple myeloma (MM). Patients with MM suffer from a generally diminished immune capacity that is likely due to the generation of a suppressive environment. Dendritic cells are central to innate and adaptive immunity because they interact with cells from both systems. DCs are pivotal for T cell priming, but tumor burden can impair DC differentiation, causing them to become dysfunctional [1]. A lack of stimulation within the tumor environment as well as defects in DC differentiation may be responsible for the low activity of the immune system [2]. In MM patients at diagnosis, the levels of myeloid DCs (mDC) and plasmacytoid DCs (pDC) are reduced compared to control donors, but normal mDC levels tend to be restored upon remission [3]. The interaction between DCs and NK cells can trigger NK cell activation. Importantly, DCs also require external stimuli to trigger NK cells [4] because immature DCs are poor inducers of interferon (IFN)-γ secretion by NK cells. Once activated, NK cells can either kill or promote DC maturation, depending on the DC/NK cell ratio [5].

Myeloma cells themselves can affect host immunity. These cells prime DCs toward a maturation state that favors the generation of T cells with regulatory rather than

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effector phenotypes [6]. In contrast to normal plasma cells, primary MM cells express CD1d antigen and can activate invariant natural killer T (iNKT) cells [7]. In MM patients, a marked polarization toward T helper cell type 2 (Th2) cytokines exists, while T helper cell type 1 (Th1) cytokines remain suppressed [8]. Both interleukin (IL)-6 and IL-10 enhance the proliferation of MM cells [9]. The MM prognostic marker β2-microglobulin is a negative regulator of the immune system, and high β2-microglobulin concentrations inhibit the generation of functional DCs in vitro [10].

A significant proportion of common chemotherapeutic agents used at sub-cytotoxic concentrations augment the stimulatory capacity of DCs [11]. A preclinical study has demonstrated that the novel pan-histone deacetylase inhibitor LBH589 decreased the production of cytokines during Toll-like receptor-induced DC activation and significantly impaired the phenotype and function of DCs [12]. Drugs can influence other cell types present in the tumor microenvironment in addition to DCs. Gemcitabine specifically reduces the number of myeloid suppressor cells found in the spleens of animals bearing large tumors [13]. The new anti-MM drug lenalidomide augments the anti-tumor effect of iNKT cells in part by increasing Th1 and reducing Th2 cytokine production [7]. Lenalidomide and thalidomide abrogate the stimulatory effect of stromal cells and significantly decrease the percentage of stem-like clonogenic MM cells [14]. The small-molecule pharmacologic agent bortezomib is a proteasome inhibitor that has received FDA approval for the treatment of MM, which has subsequently been extended to other hematologic malignancies. Proteasome inhibitors decrease the presentation of antigenic peptides and reduce tumor cell recognition by cytotoxic T cells but unexpectedly increase tumor cell recognition by NK cells [15]. The interaction between the activating NK cell receptors and their ligands represents a crucial part of the innate immune response against several malignancies, including MM. Although drug-induced potentiation of NK cell-mediated lysis is accompanied by an enhancement of ligand expression [16–18], the potentially suppressive effect of ligand upregulation on cytotoxic activity in MM should be considered [19]. The curcumin derivative FLLL32 selectively inhibits STAT3 phosphorylation and STAT3 DNA binding, reduces cell viability, and induces apoptosis in multiple myeloma and other carcinoma cancer cells with constitutively activated STAT3 signaling [20]. Recently, a synergistic apoptosis-inducing potential of rice bran arabinoxylan and curcumin was observed in the human MM cell line U266 [21].

Previous MGN-3 research has suggested that this compound may have immunomodulatory properties, mainly by acting on NK cells and enhancing their activity [22, 23]. We have shown that MGN-3 induces the maturation of human monocyte-derived DCs in vitro [24]. In this randomized placebo-controlled study, we evaluated the potential modulatory effects of MGN-3 on innate immune system parameters in patients with MM.

Materials and methods

Patients

For MM diagnoses, BM aspirates were assessed to determine the percentage of BM plasma cells by morphology and electrophoresis of serum for the presence of monoclonal immunoglobulin (Ig). The pretreatment evaluation included complete blood counts, biochemical tests for renal and liver function, and an analysis of β2-microglobulin and C-reactive protein. A radiologic skeletal survey was performed to assess the presence of bone disease. The study was approved by the University Hospital ethics committee, consistent with the Helsinki Declaration on the use of human subjects for research. The patients were diagnosed and treated at the Hospital of St. Cyril and Method (Bratislava, Slovakia). All patients gave written informed consent. A total of 48 patients with a diagnosis of MM (of which 27, i.e., 56 %, presented the IgG subtype) were evaluated before and after MGN-3 treatment in this randomized, double-blind, placebo-controlled study (one-third of recruited patients received placebo), and their baseline characteristics are summarized in Table 1. The treated patients were given alternating courses of chemotherapy based on a combination of alkylating agents (melphalan, cyclophosphamide), anthracyclines (doxorubicin, idarubicin), and glucocorticoids (dexamethasone). Patients that were positive for monoclonal Ig, exhibited less than 20 % myeloma plasma cells in the BM aspirate, and were negative for CRAB criteria did not receive chemotherapy treatment and were under observation. The patients received 2 g per day of MGN-3 granule powder or an equivalent amount of placebo dissolved in water. The contents of the placebo and MGN-3 sachets were indistinguishable in taste and appearance. The patients were monitored for 1 week before treatment to obtain the baseline values of all of the analyzed parameters, followed by 3 months of treatment. Peripheral blood (15 ml) was collected for analysis in heparin-containing tubes every 4 weeks.

MGN-3

MGN-3 is a nutritional supplement derived from rice bran hemicellulose that has been enzymatically treated with multiple hydrolyzing enzymes from Lentinus edodes mycelia (Shiitake mushrooms). The active component is an

Table 1 Baseline characteristics of patients in placebo and MGN-3 group

	MGN-3 group ($N = 32$)	Placebo group ($N = 16$)
Age, years median (range)	65 (36–82)	63 (50–80)
Sex ratio, male/female	10/22	7/9
Myeloma subtype		
IgG	17	10
IgA	7	2
IgM	1	
Light chains	2	2
Non-secretory	3	1
NA	2	1
Stage		
Durie–Salmon disease stage I/II/III	3/8/17	2/5/9
Solitary plasmacytoma	2	
NA	2	
Treatment		
Without CHT, patients under observation	11	6
CHT only before MGN-3	7	3
CHT during MGN-3	14	7

NA not analyzed, *CHT* chemotherapy

arabinoxylan that contains a xylose in its main chain and an arabinose polymer in its side chain. MGN-3 was provided by Daiwa Pharmaceuticals Co. Ltd, Tokyo, Japan.

Flow cytometry-based cytotoxicity assay

Effector peripheral blood mononuclear cells (PBMC) were isolated from the samples collected in heparin-treated tubes by Pancol (1.077 g/ml, PAN-Biotech, Aidenbach, Germany) density gradient centrifugation. The mononuclear cells from the interface were collected, washed twice with PBS, and resuspended in 10 ml of complete culture medium (CM; RPMI 1640 medium, 10 % heat-inactivated FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine) before use in the cytotoxicity assay. Target K-562 cells, an erythroleukemia cell line, were maintained in CM and split every 3–4 days. For the assays, the cells were washed once with PBS and loaded with 0.1 µM calcein probe (CAM; Molecular Probes, Eugene, OR, USA) in FCS-free RPMI medium for 15 min at 37 °C in the dark.

To determine the absolute number of viable and dead cells, equal volumes (100 µl) of Flow-Count Fluorospheres (Beckman Coulter, Brea, CA, USA) and cells were mixed and analyzed with a Coulter Epics Altra flow cytometer. The target and effector cell viability was determined by propidium iodide (PI) negativity (4 µl PI per sample, 1 mg/ml stock). Only populations with a viability of >95 % were further analyzed. The cell concentrations were calculated according to the following formula: cells/µl = $(\text{counts}_{\text{viable cells}} \times \text{concentration}_{\text{beads}})/(\text{counts}_{\text{beads}})$.

The NK cytotoxic activity in PBMCs from MM patients was determined against target K-562 cells using the CAM assay as previously described [25]. Briefly, CAM-labeled target K-562 cells were mixed with effector PBMCs to obtain six twofold serial dilutions of the *E*/*T* ratios beginning at 50:1. Triplicates of the samples and controls were seeded in 96-well V-bottomed microplates, centrifuged at $200\times g$ for 3 min, and incubated in CM for 3 h at 5 % CO_2, 37 °C. Next, the samples were transferred into cytometric tubes, and PI (4 µl of a 1 mg/ml stock per sample) was added to identify dead cells. The samples were analyzed with a Coulter Epics Altra four-color flow cytometer equipped with an argon laser operating at 488 nm. Gating was performed using side scatter (SSC; ordinate) versus the log-scale green fluorescence of the CAM probe (abscissa) to separate target cells from effector cells. To measure target cell death, green fluorescence-positive events were gated, and the PI positivity was analyzed. An average of 3,000 target cells were collected per sample. The data were analyzed with FCS Express software (De Novo Software, Los Angeles, CA, USA).

The percentage of specific lysis (PSL) was calculated at each *E*/*T* ratio as follows: % specific lysis = (CT–TE/CT) × 100 (where CT is the percentage of viable fluorescent target cells in the control tubes and TE is the percentage of viable fluorescent target cells in the experimental (target + effector) tubes). A lytic unit (LU) is defined as the number of effector cells required to lyse 20 % of a predetermined standard number (TSTD = 2×10^4) of target cells. The LU calculation was performed by fitting the curve on a semi-$\log_2$ plot of the logarithmically transformed *E*/*T* values versus the specific lysis, according to Bryant et al. [26] and Pross et al. [27]. The results are reported as the number of LUs in 10^7 effector cells.

Immunophenotypic analysis of the cells

The following mouse antihuman monoclonal antibodies (mAbs) were used to analyze mDCs, pDCs, and NK cell subsets in the peripheral blood of MM patients: CD11c-FITC, CD16-FITC, CD123-PE, CD56-PE, HLA-DR-ECD, CD45-ECD, CD3-PC5, CD14-PC5, CD16-PC5, and CD19-PC5 (Immunotech, Marseille, France). The circulating mDC and pDC subsets were defined by the concomitant lack of lineage markers ($CD3^-$, $CD14^-$, $CD16^-$, and $CD19^-$), HLA-DR expression, and mutually exclusive

membrane expression of CD11c or CD123, respectively. The results are expressed as the percentage of mDC or pDC among the HLA-DR$^+$Lin$^-$ cells. The subpopulation of NK cells was evaluated as the percentage of CD56^{dim}CD16$^+$ and CD56^{bright}CD16$^-$ cells from the CD3$^-$CD45$^+$ peripheral lymphocytes.

For immunophenotyping, whole-blood staining and a lyse/no wash method were utilized. Briefly, whole-blood aliquots (50 µl/well) were stained with 2 µl of the relevant fluorochrome-conjugated mAbs in 96-well V-bottomed microplates at room temperature for 30 min in the dark. OptiLyse B lysis (50 µl) solution was added to each well and incubated for 10 min. The samples were then transferred to cytometric tubes and further incubated with deionized H_2O (500 µl) for 10 min. The samples were measured by flow cytometry (1×10^5 events counted), and the data were analyzed with FCS Express software (De Novo Software, Los Angeles, CA, USA).

Multiplex microbead-based cytokine immunoassay

Plasma samples were prepared from heparinized peripheral blood (1 ml) by centrifugation at 5,000 rpm for 10 min. The supernatants were collected and filtered through sterile 0.22-µm-pore-size filters, and aliquots were stored at −80 °C until analysis. The plasma levels of cytokines, including IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, IFN-γ, and tumor necrosis factor (TNF)-α, were analyzed using the Bio-Plex Suspension Array System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The multiplex microbead-based cytokine immunoassay was performed in 96-well filter microplates according to the manufacturer's instructions. The cytokine standards and samples (50 µl) were diluted in plasma dilution buffer and incubated with the fluorescently labeled microspheres coupled to specific monoclonal antibodies (50 µl) for 30 min on a plate shaker (300 rpm) in the dark. After one wash step, the beads were incubated with the biotinylated detection antibody cocktail (25 µl/well) for 30 min followed by streptavidin-PE (50 µl/well) for 10 min. Finally, 125 µl of assay buffer was added to each well before reading the plate on a Bio-Plex system. The cytokine concentrations were calculated with Bio-Plex Manager Software.

Statistical analysis

Statistical significance was calculated using the SigmaPlot ver. 11 software package (Systat Software Inc., Erkrath, Germany). The paired *T* test, Wilcoxon signed rank test, or Mann–Whitney rank sum test was used for data evaluation.

Results

MGN-3 augments NK cytolytic activity in MM patients

The baseline percentage of cytolytic CD56^{dim}CD16$^+$ NK cells and cytokine-producing CD56^{bright} CD16$^-$ NK cells among the CD45$^+$CD3$^-$ peripheral lymphocytes did not differ between MGN-3- and placebo-treated patients (24.5 ± 2.8 % vs. 17.5 ± 3.5 %, $p = 0.128$; and 1.4 ± 0.2 % vs. 2.2 ± 1.0 %, $p = 0.683$, respectively). No statistically significant changes were observed in the percentages of CD56^{dim} and CD56^{bright} subpopulations of NK cells during the treatment (data not shown).

The NK cell cytolytic activity against susceptible K-562 targets was analyzed in PBMCs from MM patients receiving MGN-3 ($N = 32$) or placebo ($N = 16$) with a flow cytometry-based CAM assay. Blood samples were collected a week before treatment (baseline) and after 1, 2, and 3 months of treatment. The NK cytolytic activity was evaluated as PSL at *E/T* ratios of 50:1, 25:1, and 12.5:1 (Fig. 1a) and by calculating the number of LUs yielding 20 % cytotoxicity per 10^7 effector cells (Fig. 1b).

No statistically significant difference was found between the MGN-3 and placebo groups with regard to the baseline levels of NK activity when PSL was compared at *E/T* ratios of 50:1, 25:1, and 12.5:1 ($p = 0.297$, $p = 0.257$, and $p = 0.307$, respectively) or the LUs ($p = 0.814$). The NK cytolytic activity significantly increased above baseline levels (27 ± 4.1 % at 50:1; 17.1 ± 3.1 % at 25:1; 9.7 ± 1.9 % at 12.5:1) in the MGN-3 group at all three *E/T* ratios after 1 month (39.8 ± 5.8 %, $p \leq 0.001$ at 50:1; 25 ± 3.6 % at 25:1, $p = 0.003$; 13.9 ± 2.2 % at 12.5:1, $p = 0.008$) and 2 months of treatment (39.3 ± 5.7 %, $p = 0.038$ at 50:1; 25.3 ± 4.1 % at 25:1, $p = 0.021$; 14.4 ± 2.6 % at 12.5:1, $p = 0.044$; Fig. 1a). This increase in NK cell activity was also confirmed by comparing the lytic units; a significant increase over baseline levels (30.8 ± 7.4 LU) was observed after 1 month (47.0 ± 8.5 LU, $p = 0.045$) and 2 months (56.6 ± 12.2 LU, $p = 0.029$; Fig. 1b) of MGN-3 treatment. No significant changes in NK activity were observed in the placebo group during the treatment (Fig. 1a, b).

MGN-3 treatment increases levels of circulating myeloid DCs

The frequencies of CD11c$^+$CD123$^-$ myeloid DCs and CD11c$^-$CD123$^+$ plasmacytoid DCs as well as the mDC/pDC ratio were examined in the MGN-3-treated patients ($N = 20$) and placebo group ($N = 15$) before treatment (baseline) and after 1, 2, and 3 months of treatment (Fig. 2). There was no statistically significant difference in the percentage of mDC in the placebo and MGN-3 groups

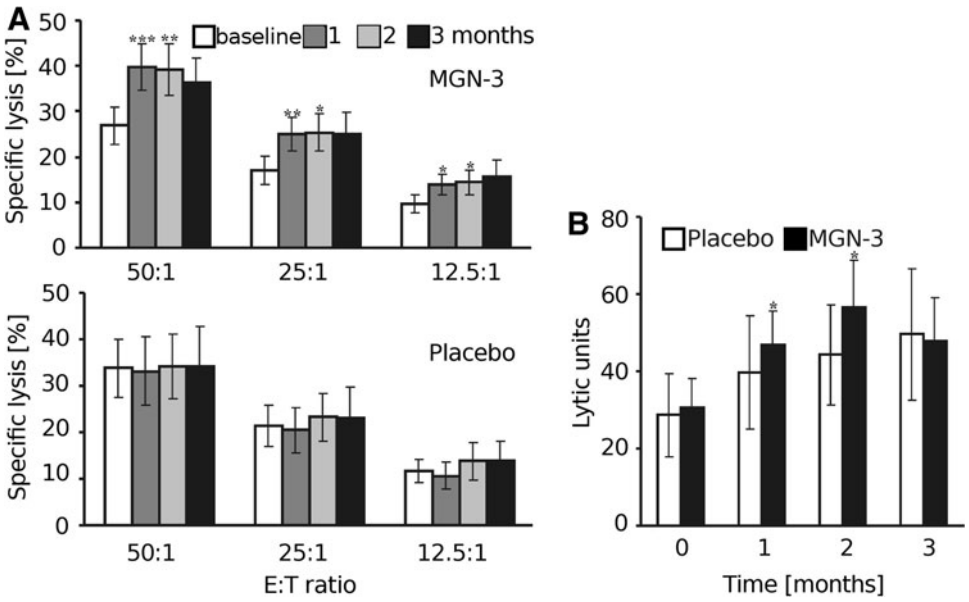

Fig. 1 Effect of MGN-3 treatment on NK cytolytic activity in MM patients. NK cell lytic activity against target K-562 cells in PBMC from MM patients receiving MGN-3 ($N = 32$) and those receiving placebo ($N = 16$) was assessed using 3-h FC-based CAM cytotoxicity assay before treatment (*baseline*), and after 1, 2, or 3 months of treatment. Statistical significance: $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ versus baseline. **a** The mean percentage of specific lysis ± SEM is shown at *E/T* ratio 50:1, 25:1, and 12,5:1. **b** Lytic units (LU, mean ± SEM) per 10^7 of effector cells

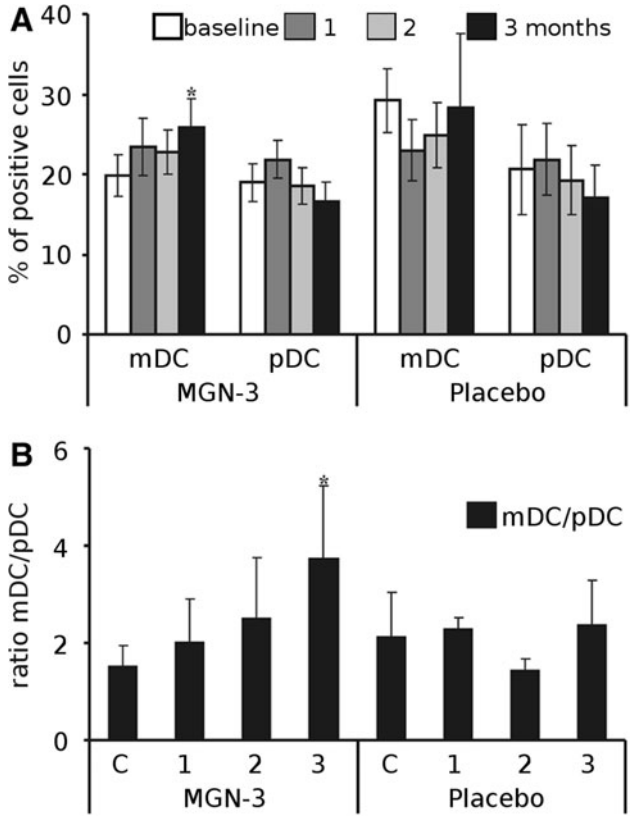

Fig. 2 Changes in circulating DC subsets after MGN-3 treatment. The levels of $CD11c^{+}CD123^{-}$ mDC and $CD11c^{-}CD123^{+}$ pDC in peripheral blood of MM patients receiving MGN-3 ($N = 20$) and placebo ($N = 15$) were analyzed by flow cytometry before treatment (*baseline*) and after 1, 2, and 3 month of treatment. $^{*}p < 0.05$, using Wilcoxon signed rank test. **a** The percentage of mDC and pDC from $HLA\text{-}DR^{+}Lin^{-}$ cells (mean ± SEM) is shown. **b** mDC/pDC ratio values (mean ± SEM) in MGN-3 and placebo group

at baseline ($p = 0.103$). The percentage of circulating mDCs among the $HLA\text{-}DR^{+}Lin^{-}$ peripheral leukocytes significantly increased after 3 months of MGN-3 treatment when compared to the baseline levels (25.8 ± 3.6 % vs. 17.6 ± 2.6 %, $p = 0.036$), while there was no significant change in the placebo group over time. The baseline values of the circulating pDCs were similar between the MGN-3 (16.6 ± 2.4 %) and placebo groups (24.9 ± 5.6 %), with no significant changes during treatment (Fig. 2a). The mDC/pDC ratio (Fig. 2b) significantly increased over time after 3 months of MGN-3 treatment ($p = 0.030$). In the placebo group, no significant changes in the mDC/pDC ratio were observed over time.

Characterization of the Th1/Th2 profile in MM patients

Patients with advanced cancer often have impaired cell-mediated immunity associated with a switch from a Thl to Th2 cytokine pattern in the local tumor environment and peripheral blood. Among the Th1 cytokines analyzed, only IFN-γ was significantly increased ($p = 0.034$) in MM patients ($N = 45$) compared to healthy controls ($N = 30$; Table 2). By contrast, we observed significant differences in the levels of six of the seven Th2 cytokines analyzed. The plasma levels of IL-4, IL-5, IL-6, and IL-13 were significantly higher in MM patients compared to healthy controls (the medians, interquartile range (IQR), and p values are presented in Table 2). However, IL-9 and IL-10 were present at significantly lower concentrations in MM patients than in healthy controls (the medians, IQR, and p values are presented in Table 2).

Table 2 Comparison of Th1 and Th2 cytokine levels between MM patients and healthy donors

	Cytokine	Healthy donors ($N = 30$)		MM patients ($N = 45$)		Difference p value
		Median (pg/ml)	IQR (pg/ml)	Median (pg/ml)	IQR (pg/ml)	
Th1	IFN-γ	44.8	16.9–74.6	82.4	24.1–137.9	0.034*
	IL-1β	1.4	0.7–2.0	1.8	0.2–2.8	0.231
	IL-2	2.4	0.0–7.6	4.2	0.0–9.3	0.175
	IL-12	3.6	2.4–9.2	3.1	0.8–6.3	0.284
	IL-15	1.5	0.0–5.2	0.7	0.0–3.1	0.550
	IL-17	0.0	0.0–3.9	1.6	0.0–4.4	0.189
	TNF-α	0.0	0.0–0.0	0.0	0.0–0.0	0.571
Th2	IL-1ra	71.6	35.6–114.3	76.4	13.9–220.1	0.837
	IL-4	0.6	0.0–0.9	0.9	0.0–2.0	0.040*
	IL-5	0.3	0.0–0.5	1.4	0.0–2.9	0.002**
	IL-6	1.9	0.0–7.3	6.4	0.0–13.0	0.027*
	IL-9	38.9	21.5–93.5	4.5	0.0–23.7	<0.001***
	IL-10	3.3	1.7–7.7	1.8	0.9–4.4	0.008**
	IL-13	1.2	0.0–2.7	4.5	0.0–23.7	0.012*

Plasmatic concentration (pg/ml) of Th1 and Th2 cytokines in MM patients ($N = 45$) and healthy donors ($N = 30$) was assessed by multiplex microbead-based immunoassay

Results are reported as medians with interquartile range (IQR; 25–75th percentile). p values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were calculated using Mann–Whitney rank sum test

HD	IL-4	IL-5	IL-6	IL-9	IL-10	IL-13
IFN-γ	57.1	137.7	12.8	0.3	9.1	34.5
IL-1β	1.5	3.7	0.3	0.0	0.2	0.9
IL-2	12.1	29.2	2.7	0.1	1.9	7.3
IL-12	8.1	19.6	1.8	0.0	1.3	4.9
IL-15	3.6	8.6	0.8	0.0	0.6	2.2
MM						
IFN-γ	66.0	53.7	14.1	6.5	31.4	6.5
IL-1β	1.2	1.0	0.3	0.1	0.6	0.1
IL-2	4.1	3.3	0.9	0.4	1.9	0.4
IL-12	2.9	2.4	0.6	0.3	1.4	0.3
IL-15	0.9	0.8	0.2	0.1	0.4	0.1

Fig. 3 Th1/Th2 cytokine ratios in healthy donors (HD) and MM patients. The values of 5 Th1 cytokines (rows: IFN-γ, IL-1β, IL-2, IL-12, and IL-15) and 6 Th2 cytokines (columns: IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13) ratios (totally 30 Th1/Th2 ratios) are shown. The ratio values were calculated using the following equation: $\text{value}_{\text{row, column}} = \text{sum}\ ((c_{\text{row}}/c_{\text{column}})_{\text{pat1}}, (c_{\text{row}}/c_{\text{column}})_{\text{pat2}}, \ldots (c_{\text{row}}/c_{\text{column}})_{\text{patN}})/N$. The values greater than 1.0 are depicted in white, the ratios less than one in *gray rectangles*

We also analyzed the Th1/Th2 ratios of 5 Th1 cytokines (IFN-γ, IL-1β, IL-2, IL-12, and IL-15) and 6 Th2 cytokines (IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13) in MM patients and healthy donors (Fig. 3). In healthy individuals, 20 of the 30 analyzed Th1/Th2 ratios were greater than 1.0, and only 10 were less than 1.0 (Th1/Th2 score 20:10). In MM patients, 14 Th1/Th2 ratios were greater than 1.0, and 16 were less than 1.0 (Th1/Th2 score 14:16), which suggests that Th2 immunity may be predominantly active in patients with MM. Specifically, a shift toward the Th2 pattern was observed for the IL-12/IL-6, IL-12/IL-13, IL-2/IL-6, IL-2/IL-13, IL-15/IL-4, IL-15/IL-5, and IL-15/IL-13 ratios of MM patients when compared to healthy donors.

Effect of MGN-3 treatment on Th1 and Th2 cytokine levels

We also assessed the plasma concentrations of Th1 and Th2 cytokines in patients treated with MGN-3 ($N = 30$) or placebo ($N = 15$; Fig. 4). No statistically significant differences were observed between the groups when the baseline values of Th1 and Th2 cytokines were compared. After 1 month of MGN-3 treatment, we observed significantly elevated levels of IL-1β ($p = 0.047$), IL-12 ($p = 0.011$), IL-17 ($p = 0.036$), and TNF-α ($p = 0.01$) compared to the baseline levels before treatment (Fig. 4a). After 3 months, we observed increased levels of IL-12 ($p \leq 0.001$), IL-17 ($p = 0.032$), IFN-γ ($p = 0.018$), and TNF-α ($p = 0.007$) in the MGN-3 group (Fig. 4a).

Among the Th2 cytokines, the levels of IL-5 and IL-9 were significantly increased in the MGN-3 group ($p = 0.014$ and $p = 0.031$, respectively). However, IL-10 levels were significantly reduced in the placebo group after 1 month of treatment ($p = 0.037$), and IL-4 levels were elevated in both the MGN-3 and placebo groups compared

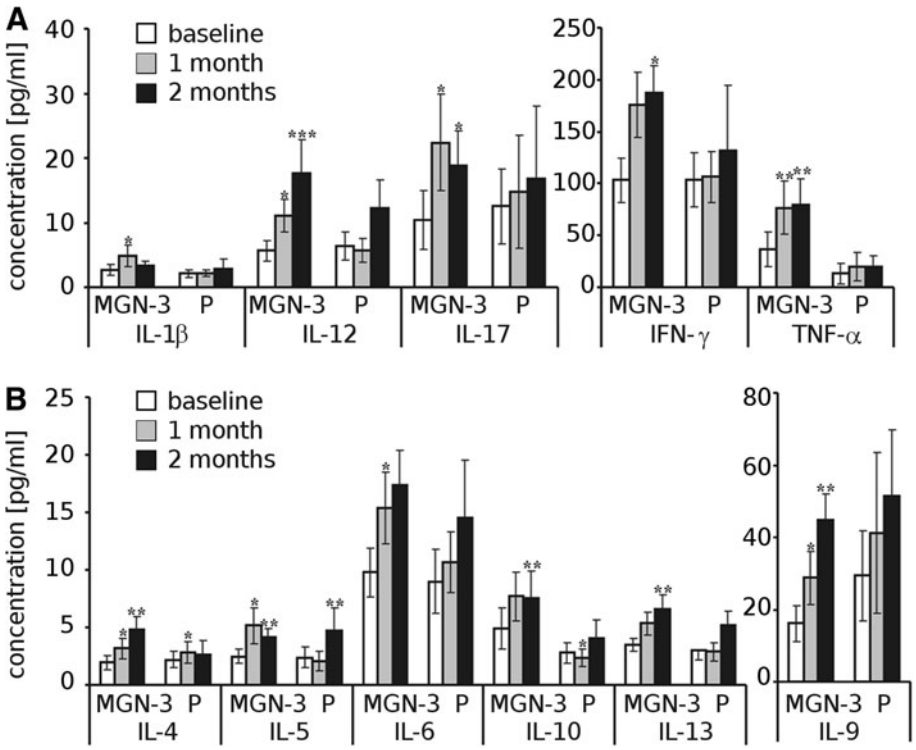

Fig. 4 Changes in Th1 and Th2 cytokine profile after MGN-3 treatment. Plasmatic concentration (pg/ml) of a Th1 cytokines and IL-17 and b Th2 cytokines was measured using multiplex microbead-based immunoassay in patients treated with MGN-3 ($N = 30$) or placebo ($N = 15$). Statistical significance: $*p < 0.05$; $**p < 0.01$, $***p < 0.001$

to the baseline levels ($p = 0.034$ and $p = 0.024$, respectively). After 3 months of MGN-3 treatment, IL-4 ($p = 0.003$), IL-6 ($p = 0.031$), IL-9 ($p = 0.006$), IL-10 ($p = 0.010$), and IL-13 ($p = 0.002$) levels were significantly increased compared to the placebo, whereas IL-5 levels increased in both the MGN-3 and placebo groups ($p = 0.006$ and $p = 0.004$, respectively; Fig. 4b).

Discussion

Recent evidence suggests that NK cells play an important role in MM immunosurveillance by exerting anti-MM cytotoxicity. The differential expression of NK cell surface receptors and their ligands on myeloma cells during disease progression reflects immune editing and the selection of more aggressive myeloma clones that are resistant to NK-mediated lysis [19, 28, 29]. The effect of anti-MM cytotoxicity can be enhanced by novel therapies, such as the adoptive transfer of in vitro-activated NK cells with increased anti-myeloma activity [30] or comprehensive treatment with proteasome inhibitors [31] and immunomodulatory drugs [32, 33], which have been demonstrated to increase NK cell numbers and function.

We evaluated NK cell activity in the peripheral blood of MM patients during treatment with MGN-3. Consistent with the previously published data [22], we observed a statistically significant increase in NK-mediated cytotoxicity during the first 2 months of MGN-3 treatment, while no statistically significant differences in NK cytotoxicity were detected in the placebo-controlled group. However, the phenotypic analysis performed in MM patients did not reveal changes in the percentage of NK cell subsets. This effect is similar to the results observed in humans during treatment with other types of natural products [34–36].

In our previous in vitro study, we demonstrated that MGN-3 augmented the maturation of monocyte-derived DCs and induced a pDC-like phenotype switch [24]. Dendritic cells are a key component of the immune system and play a critical role in priming naïve T cells and inducing tumor-specific protective immune responses [37]. Patients with MM suffer from general impaired immunity involving deficiencies in DC frequencies and functions. Both the mDC and pDC subsets of DCs were significantly reduced in the peripheral blood of MM patients compared to healthy age-matched controls [3, 38, 39]. In addition, DCs in MM patients exhibited phenotypic abnormalities as well as an altered pattern of inflammatory cytokine secretion [39–41]. Our phenotypic analysis of the peripheral blood from MM patients revealed a significant increase in the relative concentration of mDCs. Similarly, an increase in the mDC/pDC ratio was observed after MGN-3 treatment but not in the placebo group, while there were no significant changes in circulating pDC levels in either group. Because our study was limited to peripheral blood analysis, further studies are needed to determine whether

the phenotypic pDC switch observed in vivo and the increase in mDCs in peripheral blood is due to the preference of pDCs for bone marrow [42].

Immune DC dysfunction has been linked to high levels of soluble factors, including multiple cytokines, such as VEGF, IL-6, and IL-10, which interfere with DC differentiation and maturation [43, 44]. As mediators of the immune response, cytokines are often classified as Th1-type cytokines, which mainly induce cell-mediated immunity, and Th2-type cytokines, which predominantly induce humoral immunity [45].

We analyzed the concentrations of Th1 and Th2-related cytokines in the plasma of MM patients compared to healthy donors. The plasma cytokine levels and Th1/Th2 profiles observed in healthy controls were consistent with the published data [46, 47]. In MM patients, we observed a statistically significant increase in Th1-related IFN-γ along with increases in the Th2 cytokines IL-4, IL-5, IL-6, and IL-13, while levels of IL-9 and IL-10 were significantly lower in MM patients compared to healthy controls. These results indicate deregulated immune homeostasis and a shift toward systemic Th2 cytokine dominance, as previously described in patients with cancers including MM [8].

Along with Th1 cytokines, an increased concentration of IL-1(α,β) and IL-6 is consistently associated with successful anti-myeloma immunosurveillance [48] and possible cancer eradication. In our study, we observed increased levels of several important Th1 cytokines, in particular IL-1β, IL-12, IFN-γ, and TNF-α, in MM patients receiving MGN-3, in contrast to the placebo group. In addition, the concentration of IL-17 was significantly increased upon MGN-3 treatment. Very recently, a similar pattern of in vitro production of proinflammatory and immuno-regulatory cytokines in DCs stimulated by MGN-3 was observed to induce CD4 + cell proliferation, and the production of IFNγ, IL-10, and IL-17 was observed [49].

The study results demonstrate a clear increase in NK activity, an increased level of mDCs in the peripheral blood, and augmented levels of Th1-related cytokines in the plasma of MM patients treated with MGN-3 compared to the placebo group. To elucidate the underlying mechanisms, the immunomodulatory effects of MGN-3 merit further study involving focused attention on the bone marrow environment.

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