Both mucosal-associated invariant and natural killer T-cell deficiency in multiple myeloma can be countered by PD-1 inhibition

Mucosal associated invariant T (MAIT) cells primarily contribute to immune defense against infectious pathogens and regulate pathogenesis of various inflammatory diseases.[1] They are innate-like T lymphocytes expressing an invariant TCR Vα7.2-Jα33 chain in humans and displaying high expression levels of CD161 and IL-18R. They are primarily localized in mucosal tissue and respond to vitamin B2 metabolites in an MHC class 1b (MR1)-dependent manner.[1] Despite a different ontogeny, MAIT cells share a close lineage relationship with invariant natural killer T (iNKT) cells, another invariant T-cell subset recognizing glycolipids and important for anti-tumor immunity.[1,2] Moreover, it has been suggested that they share a common niche and could therefore be functionally redundant.[3] Recently, MAIT cells have been implicated in cancer and were shown to support anti-tumor immunity;[4] however, their exact role has not been well explored. Until now, most reports on involvement of MAIT cells in cancer have been limited to mucosa-associated cancers.[5] Recently, however, Wallace et al. reported MAIT cell deficiency in chronic lymphocyte leukemia (CLL), suggesting a possible involvement of MAIT cells in hematologic malignancies as well.[4] Further investigation of MAIT cell functionality in cancer patients beyond mucosa-associated malignancies is, therefore, warranted. In multiple myeloma (MM), invariant T cells such as iNKT cells have been reported by us and others to be deficient.[6] This has not yet been investigated for MAIT cells and whether they prove to be

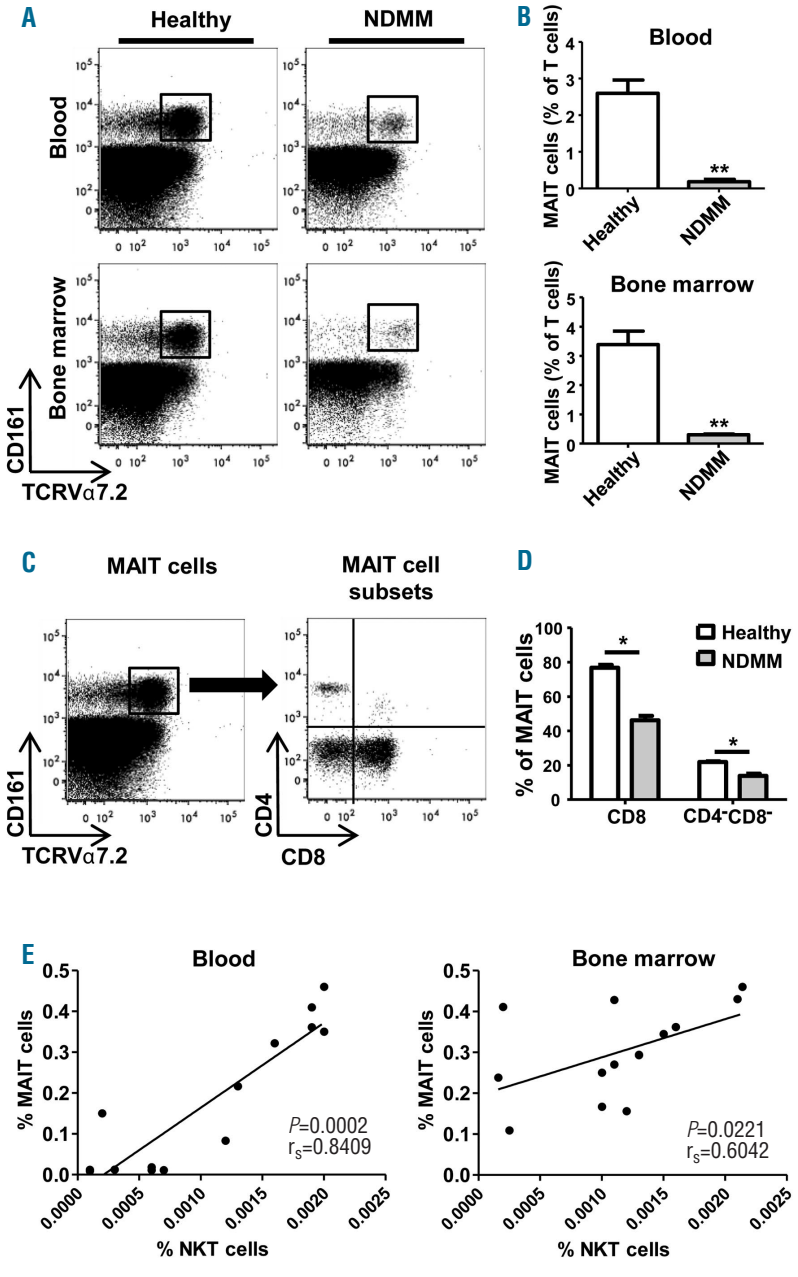

Figure 1. MAIT-cell numbers are decreased in newly diagnosed multiple myeloma (NDMM) patients compared to healthy controls and correlate with decreased invariant natural killer T-cell (iNKT) numbers. (A) Flow cytometry data plots illustrating the MAIT-cell gating strategy in the peripheral blood and bone marrow (BM) of healthy controls (n=14) and NDMM patients (n=14). (B) MAIT-cell percentages within peripheral blood and BM T cells in healthy controls and NDMM patients. (C) Representative MAIT-cell subsets as determined by flow cytometry and (D) $CD8^+$ and $CD4^-CD8^-$ MAIT-cell percentages within peripheral blood and BM T cells in healthy controls and NDMM patients. (E) Spearman's correlation analysis between MAIT-cell levels and NKT-cell levels in blood and BM of NDMM patients. *P<0.01, **P<0.001.

important in MM has not been established. Their similarities with iNKT cells and their regulatory functions, together with recent reports suggesting their possible involvement in non-mucosa-associated malignancy, make them a logical candidate for further studies. Therefore, we examined the phenotype and functionality of MAIT cells in MM.

A total of 14 newly diagnosed MM (NDMM) patients and 14 age-matched healthy controls were enrolled in this study (patients' clinical characteristics are available in *Online Supplementary Table S1*). The frequency of MAIT cells ($CD3^+V\alpha7.2^+CD161^+$) in total $CD3^+$ lymphocytes was determined by flow cytometry in peripheral blood (PB) and bone marrow (BM) of NDMM patients and healthy counterparts (Figure 1A). MAIT cell percentages were significantly lower in NDMM patients compared to

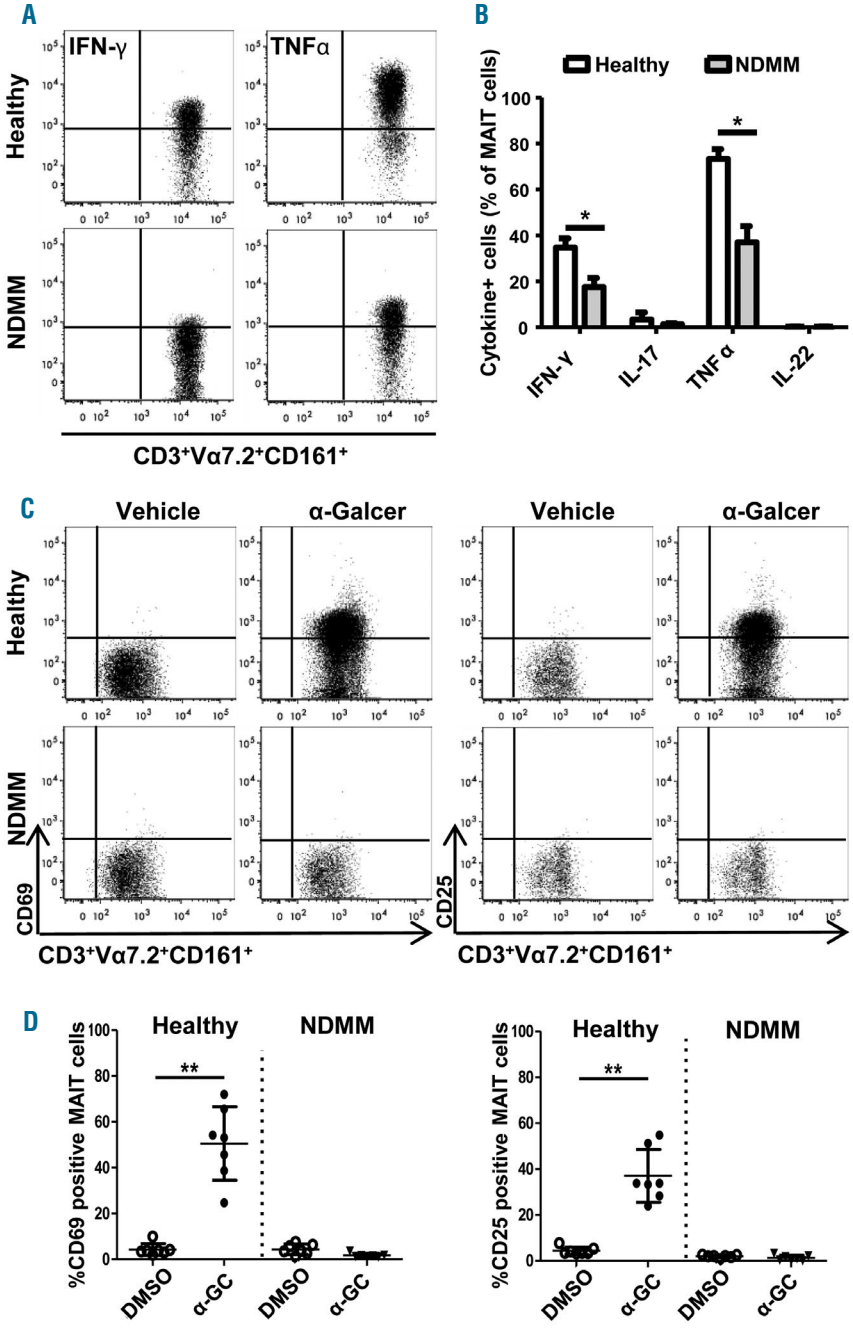

Figure 2. Impaired functionality of MAIT cells in newly diagnosed multiple myeloma (NDMM) patients compared to healthy controls. (A) Representative IFN-γ, IL-17, and TNFα expression levels in the MAIT-cell population of healthy individuals (n=7) and NDMM patients (n=7), as determined by intracellular flow cytometry after stimulation with PMA and ionomycin. (B) Percentages of IFN-γ, IL-17, IL-22 and TNFα levels produced by MAIT cells in response to PMA and ionomycin are illustrated. (C) Flow cytometry data plots of CD69 and CD25 expression levels within the MAIT-cell population of healthy donors (n=7) and NDMM patients (n=7) after three days of stimulation with or without α-GalCer. (D) Percentage CD69 and CD25 positive NKT and MAIT cells after three days of stimulation with or without α-GalCer. DMSO: DMSO vehicle; α-GalCer: alpha-galactosyl ceramide. *P<0.05, **P<0.001.

healthy individuals (Figure 1B). Reduced circulating MAIT cell numbers have also been reported in autoimmune disorders [e.g. systemic lupus erythematosus (SLE), rheumatoid arthritis and multiple sclerosis] and infectious diseases (tuberculosis, HIV) along with malignancies (chronic lymphocytic leukemia and mucosa-associated cancers).[4,5,7-11] However, we are the first to show MAIT cell disturbances in MM. MAIT cells can be subdivided into CD4$^+$, CD8$^+$ and double negative subsets (Figure 1C). In PB and BM, most of the MAIT cells carry either a CD8$^+$ or a double negative phenotype. Analysis of MAIT cells in NDMM patients by flow cytometry revealed significantly lower percentages of the CD8$^+$ MAIT cell subset and the double negative fraction (Figure 1D), while the CD4$^+$ fraction remained unchanged (*data not shown*). Therefore, reduced CD8$^+$ and DN subset levels seem to contribute to total MAIT-cell number reduction in MM. Similar observations were published for SLE, HIV and multiple sclerosis, but have not yet been reported in other cancers.[8,10,11]

In addition, similar decreases of iNKT-cell numbers in MM could be observed (*Online Supplementary Figure S1A* and *B*), in line with earlier reports.[6] Since MAIT and iNKT cells share a close lineage relationship, we subsequently

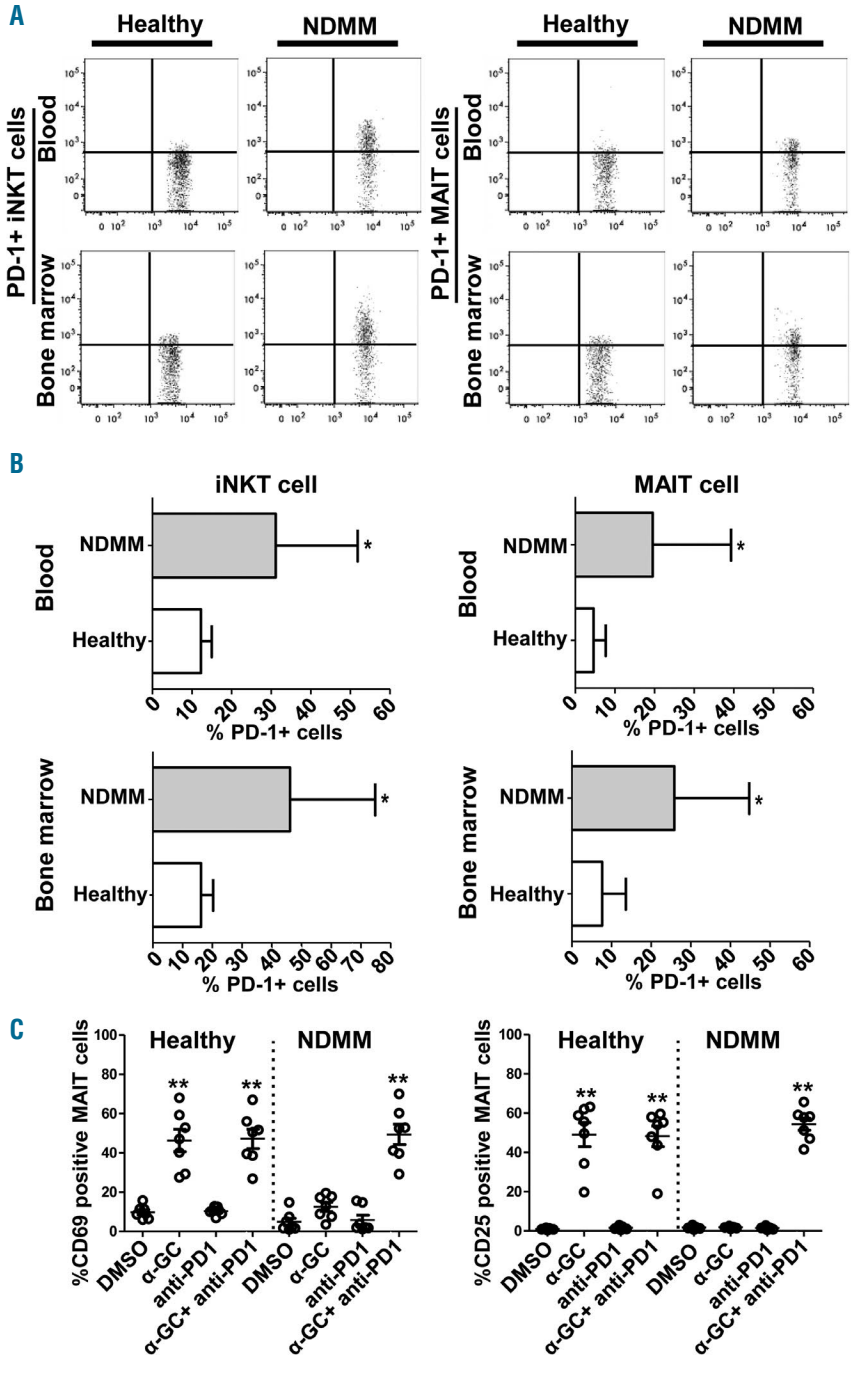

Figure 3. Increased PD-1 levels on MAIT and iNKT cells in newly diagnosed multiple myeloma (NDMM) patients and restoration of MAIT-cell activation by invariant natural killer T-cell (iNKT) stimulation and PD-1 blockade *in vitro*. (A) Flow cytometry data plots illustrating PD-1 expression levels on MAIT and iNKT cells in the peripheral blood and bone marrow (BM) of healthy controls (n=14) and NDMM patients (n=14). (B) Percentages of PD-1 positive NKT and MAIT cells among peripheral blood and BM in healthy controls (n=14) and NDMM patients (n=14). (C) Percentage of CD69 and CD25 positive cells within the MAIT-cell population of healthy donors (n=7) and NDMM patients (n=7) after three days of stimulation with or without α-GalCer and anti-PD-1. DMSO: DMSO vehicle; α-GalCer: alpha-galactosyl ceramide; programmed death 1 checkpoint molecule (PD-1). *P<0.05, **P<0.001.

evaluated a potential relationship between them in NDMM patients, by analyzing the association between the MAIT-cell levels and iNKT-cell levels (Figure 1E). Spearman's correlation analyses revealed a significant correlation between MAIT cell percentages and the total iNKT-cell levels in NDMM patients, suggesting that MAIT-cell deficiency is linked to iNKT-cell impairment.

Next, we evaluated the cytokine profile of MAIT cells in order to determine their functionality in NDMM. Peripheral blood mononuclear cells (PBMCs) from 7 NDMM patients and 7 healthy controls were stimulated for 4 hours in the presence of PMA and ionomycin. Subsequently, expression levels of IFN-γ, IL-17, IL-22 and TNFα were examined by flow cytometry (Figure 2A). Expression of IFN-γ, IL-17, IL-22 and TNFα was absent on unstimulated MAIT cells of both patients and healthy individuals (*data not shown*). However, when stimulated, percentages of IFN-γ and TNFα positive MAIT cells were found to be significantly reduced in PBMCs derived from NDMM patients compared to healthy controls (Figure 2B). IL-17 and IL-22 MAIT cell expression levels were unchanged between NDMM patients and healthy donors (Figure 2B). As described in other studies, the majority of MAIT cells (80-90%) produce TNFα and IFN-γ compared to a minority (3% on average) of IL-17 and IL-22–producing MAIT cells.[2] Together, these data suggest that MAIT cells of NDMM patients have a disturbed Th1 function. This could either be due to reduced production of Th1 cytokines or to polarization of their cytokine profile.

In order to assess whether iNKT cells have the capacity to activate MAIT cells, PBMCs from 7 NDMM patients and 7 healthy controls were incubated for 72 hours in the presence or not of α-GalCer, a strong glycolipid agonist for iNKT cells. Subsequently, CD69 and CD25 expression was determined in the MAIT-cell population by flow cytometry (Figure 2C). In healthy subjects, marked increases of $CD69^+$ and $CD25^+$ MAIT cells could clearly be observed after stimulation with α-GalCer in comparison with vehicle conditions (Figure 2D). In contrast, percentages of $CD69^+$ and $CD25^+$ MAIT cells were markedly reduced upon iNKT stimulation in NDMM patients. These findings suggest that a dysfunction of iNKT cells, MAIT cells or a combination of both could be responsible for a reduced activation of MAIT cells in NDMM patients, as has also been observed in SLE.[10] Altogether, this further supports the intriguing concept that dysfunctional iNKT-MAIT cell interactions can be involved in pathological conditions. Although the underlying mechanisms of crossregulation between iNKT and MAIT cells are still not unknown, it can be anticipated that iNKT-MAIT cell communication is partly cytokine mediated. However, it should also be noted that iNKT-cell activation leads to bystander stimulation of a broad range of downstream effector cells, including NK cells which could potentially also contribute to MAIT cell activation.[1] This will be the subject of future research.

PD-1 is a well-known target for immune checkpoint inhibition in cancer, as tumor cells are able to evade the immune system by PD-1 – PDL1/2 signaling. In conventional T cells, PD-1 is absent on naïve T cells but up-regulated after T-cell activation. Recent reports highlighted PD-1 and its ligand PD-L1/2 as being implicated in induction and maintenance of iNKT-cell anergy.[12-14] We, therefore, investigated whether the observed iNKT- and MAIT-cell dysfunction was related to aberrant PD-1 expression as determined by flow cytometry (Figure 3A). Interestingly, PD-1 levels were increased both on iNKT and MAIT cells in BM and PB of NDMM patients (n=14) as compared to healthy controls (n=14) (Figure 3B). Comparable results were observed in the 5T33MM murine model (*Online Supplementary Figure S2A*). We next assessed the impact of PD-1 blockade on MAIT cell function in NDMM patients by performing co-culture experiments *in vitro*. PBMCs from 7 NDMM patients and 7 healthy controls were incubated for 72 hours in the presence or not of α-GalCer with or without PD-1 blockade. Remarkably, activation of MAIT cells by α-GalCer-stimulated iNKT was recovered in NDMM patients in the presence of PD-1 blockade compared to the condition with α-GalCer and PD-1 block alone (Figure 3C). Similar immune activation by α-GalCer together with PD-1 blockade was found in the 5T33MM model (*Online Supplementary Figure S2B*). Successful re-activation of MAIT cells by PD-1 blockade suggests that the PD-1 pathway is one of the contributors mediating MAIT-cell dysfunction in MM and disturbing MAIT–iNKT immune interactions. Moreover, PD-1 blockade combined with α-GalCer stimulation led to a strong reduction in tumor load *in vivo* in the 5T33MM model (*Online Supplementary Figure S3*).

To the best of our knowledge, we are the first to demonstrate that MAIT cells are numerically and functionally impaired in NDMM patients. In addition, this was found to be linked with iNKT-cell deficiency and elevated PD-1 levels. PD-1 blockade together with α-GalCer-stimulated iNKT cells rescued this deficiency and conferred tumor protection in the 5T33MM murine model. These results open doors for further studies and stimulate research to elucidate the exact role of MAIT cells in MM. Apart from evaluating MAIT cells in other MM patient subsets (monoclonal gammopathy of unknown significance, smoldering MM and relapse), long-term studies in large cohorts of patients will allow us to evaluate if these alterations can be linked to clinical outcome. Furthermore, it is of interest to evaluate MAIT- and iNKT-cell levels in anti-PD-1-treated MM patients. However, we believe that the impact of MAIT cells in the MM microenvironment, as well as improvement of their effector functions *via* immune checkpoint blockade, represents a relevant and attractive field for immunosurveillance and immunotherapy in MM. In line with Richter *et al.*, demonstrating clinical regression after combining lenalidomide and iNKT-cell stimulation in MM patients, we provide supplementary evidence for harnessing invariant T cells to prevent MM.[15] Therefore, targeting invariant T cells which simultaneously stimulate both innate and adaptive immunity, together with PD-1 blockade, might provide a more broad immune activation, and could, therefore, give more advantageous results compared to current ongoing trials.

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