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

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SUPPLEMENTARY MATERIALS AND METHODS

5T33MM mice

C57BL/KaLwRijHsd mice were purchased from Envigo (Horst, The Netherlands). Housing, maintenance and experiments were performed following regulations approved by the Ethical Committee for Animal Experiments, Vrije Universiteit Brussel (CEP n° 14_281_12). The 5TMM murine models have been previously described [1–3].

Myeloma Patients

After informed consent, BM and peripheral blood were collected from 14 untreated NDMM patients and age matched healthy controls. Patient characteristics are summarized in supplementary Table 1. The study was conducted in accordance with principles of the Declaration of Helsinki. Research was approved by the Ethic Board of UZ Brussel (B.U.N. 143201316382) and Tumourbank of Lille (CSTMT102).

Flow Cytometry and antibodies

Immunophenotyping was determined by flow cytometry using fluorochrome-conjugated monoclonal antibodies (mAbs). Analyses were performed using FlowJo software (Tree Star Inc., Ashland, OR).

Peripheral blood and BM samples from 14 healthy donors and 14 NDMM patients were collected in heparin-containing tubes. Human iNKT and MAIT cells were identified phenotypically as previously reported[4–7]. Mononuclear cells were obtained after Ficoll density gradient centrifugation (Histopaque, Sigma-Aldrich) and stained with PE-conjugated anti-human Vα24Jα18 TCR (6B11), PERCP Cy5.5-conjugated anti-CD161 (HP-3G10), Amcyan-conjugated anti-CD3ε (UCHT1), Pacific Blue-

conjugated anti-CD4 (OKT4), Alexa Fluor 700-conjugated anti-CD8 (OKT8), PE-eFluor 610-conjungated anti-PD-1 (J43) (all eBioscience, San Diego, CA), Brilliant Violet 605-conjungated anti-TCRVα7.2 (3C10, Biolegend, San Diego, CA) and acquired on a FACSLSRII (Becton Dickinson). Intracellular cytokine flow cytometry was performed to determine the expression levels of IFN-γ, IL-17, TNFα and IL-22 in MAIT cells of NDMM patients and healthy controls. MAIT cells were stained with PERCP Cy5.5-conjugated anti-IFN-γ, PERCP Cy5.5-conjugated anti-IL-17, PE-conjugated anti-TNFα, and PE-conjugated anti-IL-22 (all eBioscience) and analyzed by flow cytometry (FACSCanto II; Becton Dickinson). For the analysis of activation markers, APC-conjugated CD69 (FN50) and PE-Cy7-conjugated CD25 mAbs (BC96, both eBioscience) were used.

Murine liver, blood, spleen and BM cells were isolated as previously described[1,8]. Lymphocytes were isolated and resuspended in staining buffer containing anti-Fcγ Receptor type II/type III monoclonal antibodies (Miltenyi Biotec). Cells were stained with PE-labelled CD1d/α-GalCer tetramer, FITC-conjugated anti-279 (PD-1, J43), APC-Cy7-conjugated anti-TCRβ (H57-597), PE-Cy7-conjugated anti-CD3 (145-2C11), Pacific Blue-conjugated anti-NK1.1 (PK136, all eBioscience), Amcyan-conjugated anti-CD4 (RMA4-5) and 7-AAD (Both Beckton Dickinson) and living cells were acquired on a FACSCantoII (BD Biosciences) flow cytometer.

Functional MAIT cell assays

PBMCs (1,5 x10^6/ well) were incubated in complete RPMI 1640 with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS (Life technologies BRL), stimulated with Phorbol-Myristate-Acetate (PMA, 100 ng/ml;

Sigma-Aldrich), ionomycin (1 µM; Sigma-Aldrich) and brefeldin A (GolgiPlug; 10 µg /ml; BD Biosciences) for 4 hours and subsequently stained for MAIT markers. After fixation in 4% paraformaldehyde and permeabilization with Perm/Wash solution (BD Biosciences), MAIT cells were intracellularly stained for IFN-γ, IL-17, TNFα and IL-22 and analyzed by flow cytometry.

PBMCs (1,5 x 10^6/ well) were stimulated with α-GalCer (100 ng/ml; Department Chemistry; Ghent University) or 0,1% DMSO (vehicle) and incubated with or without anti-human anti-PD-1 Ab (5 µg/ml, eBioscience) for 3 days in the presence of IL-2 (100 U/ml, Roche). After 72 hours, PBMCs were stained and analyzed by flow cytometry for MAIT cells and iNKT cells markers next to the activation markers CD69 and CD25.

NKT-MM co-culture assay

Isolation of 5T33MMvv, 5T2MM, iNKT and dendritic cells (DCs) were performed as previously described[1,8,9,10]. 5x10^4 5T33MMvv / 5T33MMvt / 5T2MM cells, 5x10^4 iNKT cells, 10^5 α-GalCer loaded or vehicle loaded DCs were co-cultured in triplicate in presence or absence of murine anti-PD-1 Ab (5 µg/ml, Bioceros, The Netherlands). After 24 hours supernatants of co-cultures were collected for IFN-γ measurements by ELISA (eBioscience), following manufacturer's instructions.

In vivo treatment with α-GalCer and anti-PD-1 in MM

Naive C57BL/KaLwRijHsd mice were i.v. injected with 5 x 10^5 5T33MM cells and i.p. injected with 200 µg murine anti-PD-1 Ab and 2 µg α-GalCer. α-GalCer injections were repeated weekly (day 6, day 13) and anti-PD-1 Ab was given twice a week.

Serum IFN-γ levels were determined by ELISA, 16 hours after i.p. α-GalCer administration (day 0 and day 6). Mice were sacrificed when first signs of morbidity appeared (in general at day 21). Serum M–spike levels were determined by protein electrophoresis and cytosmears were processed to evaluate BM tumor load.

Statistics

Results are expressed as mean ± SEM. Spearman's Correlation, one-way ANOVA with Bonferroni correction and Mann-Whitney U-tests were used to address statistical significance.

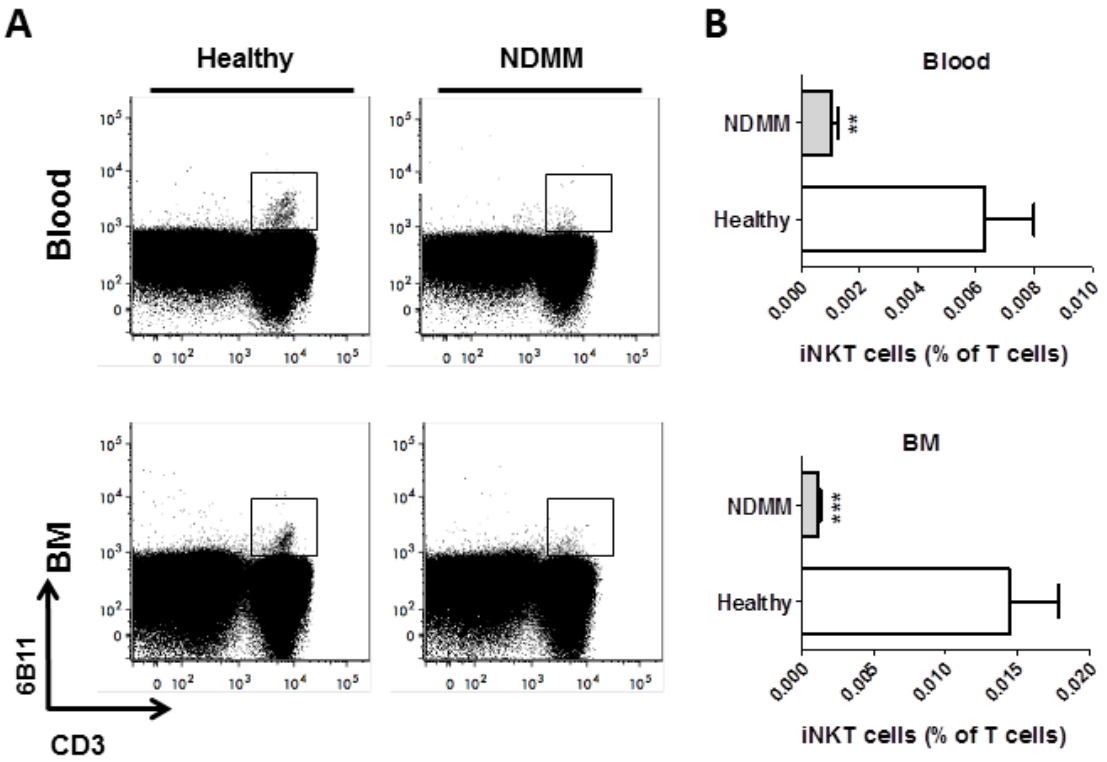

Figure S1: Decreased iNKT cell numbers in NDMM patients compared to healthy controls. A) Flow cytometry data plots illustrating the iNKT cell gating strategy in the peripheral blood and BM of healthy controls (n=14) and NDMM patients (n=14). B) iNKT cell percentages within peripheral blood and BM T cells in healthy controls and NDMM patients. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

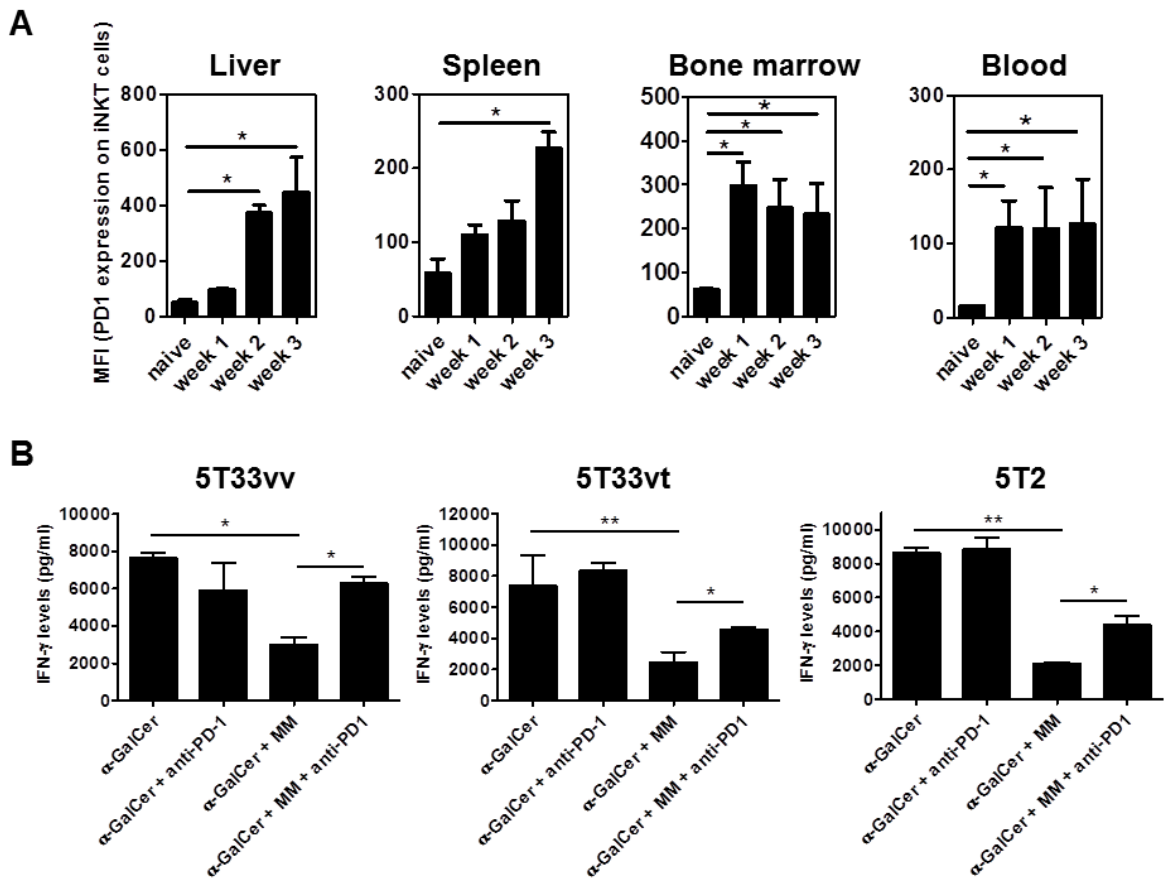

Figure S2: Increased PD-1 expression on iNKT cells in the 5T33MM murine model and restoration of iNKT functionality by PD-1 blockade in vitro. A) Percentages PD-1 positive NKT cells in liver, spleen, BM, blood of the 5T33MM murine model. B) Mean IFN-γ levels of murine iNKT co-culture experiments are illustrated. The experiment was performed with 5T33vv, 5T33vt and 5T2 MM cells. Respective conditions are iNKT cells + α-GalCer-loaded DC's; iNKT cells + α-GC-loaded DC's + anti-PD-1; iNKT cells + α-GC- loaded DC's + MM cells; iNKT cells + α-GC- loaded DC's + MM cells + anti-PD-1. Vivo (vv); Vitro (vt); Alpha-Galactosylceramide (α-GalCer); Multiple myeloma cells (MM). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

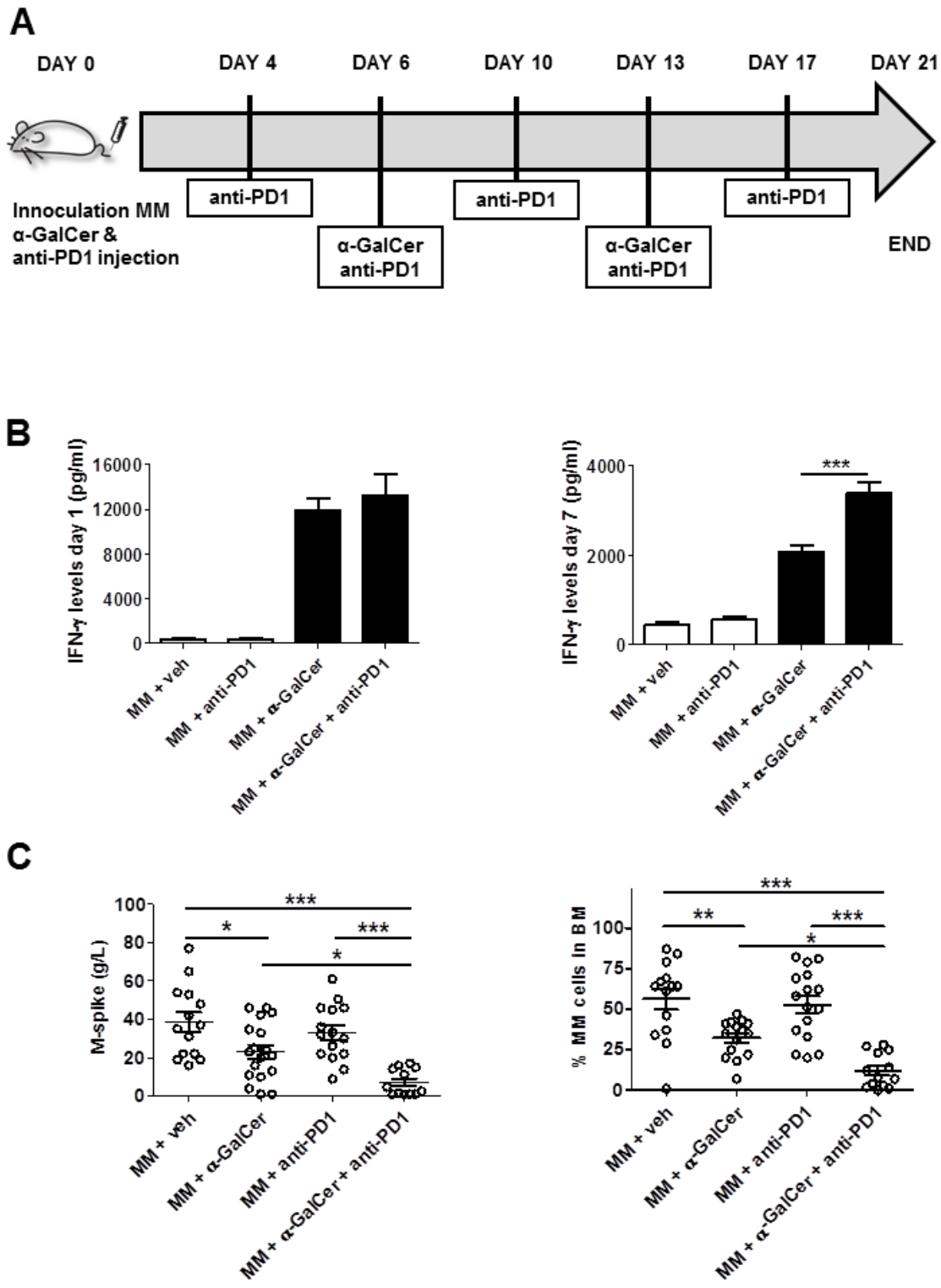

Figure S3: In vivo treatment of PD-1 blockade and α-GalCer restored tumor immunity in the 5T33MM murine model. A) Schematic overview of the experiment. C57BL/KaLwRij mice were inoculated together with 2µg α-GalCer and 200µg anti-PD-1 at day 0. α-GalCer was re-injected weekly at day 6 and day 13. Injections with

anti-PD-1 were given twice a week. Treatment groups were MM + veh (n=14), MM + anti-PD-1 (n=16), MM + α-GalCer (n=15), MM + α-GalCer + anti-PD-1 (n=13). B) Serum M-spike levels and percentage malignant plasma cell in the BM. C) IFN-γ levels in the serum 16h after the first stimulation and second stimulation (one week later) with α-GalCer. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Supplementary table 1: Characteristics healthy controls and NDMM patients

	Number	Mean	Range
Control individuals (n= 14)			
Age (years)		62	54-76
Gender (M/F)	8/6		
Newly Diagnosed multiple myeloma (n= 14)			
Age (years)		70	57-81
Gender (M/F)	8/6		
M- component type			
IgG	10		
IgA	3		
IgM	1		
IgD	-		
Light chain type			
kappa	7		
lambda	7		
ISS Stage[1]			
Stage I	4		
Stage II	6		
Stage III	4		
Adverse cytogenetics			
13q	5		
t(11,14)	9		
17p	2		

[1]**ISS: International Staging System**

SUPPLEMENTARY REFERENCES

1 Vanderkerken K, Croucher P. Multiple myeloma biology : lessons from the 5TMM models. Immunol Rev 2003;194:196–206.

2 Radl J, Glopper E De, Schuit HRE, et al. Idiopathic Paraproteinemia : II . Transplantation of the Paraprotein-Producing Clone from Old to Young C57BL / KaLwRij Mice. J Immunol 1979;122:609–613.

3 Vanderkerken K, Asosingh K, Willems A, et al. The 5T2MM murine model of multiple myeloma: maintenance and analysis. Methods Mol Med 2005;113:191–205.

4 Le Bourhis L, Martin E, Péguillet I, et al. Antimicrobial activity of mucosal-associated invariant T cells. Nat Immunol 2010;11:701–708.

5 Martin E, Treiner E, Duban L, et al. Stepwise development of mait cells in mouse and human. PLoS Biol 2009;7:0525–0536.

6 Venken K, Melis L, Jacques P, et al. Synovial fluid derived invariant natural killer T cells in chronic arthritides show an increased programmed death-1 expression and anergic phenotype. Ann Reum Dis 2013;71.

7 Metelitsa LS. Flow cytometry for natural killer T cells: Multi-parameter methods for multifunctional cells. Clin Immunol 2004;110:267–276.

8 Jacques P, Venken K, Beneden K Van, et al. Invariant Natural Killer T Cells Are Natural Regulators of Murine Spondylarthritis. 2010;62:988–999.

9 Lutz MB, Kukutsch N, Ogilvie ALJ, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. 1999;:77–92.

10 Govindarajan, Srinath Elewaut D, Drennan M. An Optimized Method for Isolating and Expanding Invariant Natural Killer T Cells from Mouse Spleen. J Vis Exp 2015;105:doi: 10.3791/53256.