Supplementary results

ELISPOT Assay

The specific recognition of TAAs and/or HLA-matched allogeneic melanoma cell lines by *ex vivo* isolated PBMCs was determined using an IFN-γ-release assay (ELISPOT) (*Parmiani G. et al. OncoImmunol, 2015*). Anti-human IFN-γ mAb (1-D1K), the secondary biotin-conjugated anti-IFN-γ mAb (7-B6-1) and the secondary alkalin phosphatase-streptavidin were purchased from Mabtech (Naka Stand, Sweden). PBMCs (3 x 10^4 cells/well) were incubated in flat bottom 96-well plates in the presence of 1.7 x 10^4 target cells/well. The cell lines T2 (HLA-2^+), 1061 EBV-B (HLA-$A1^+$) or 1869 EBV-B (HLA-$A3^+$ and $A24^+$) cells were used as antigen presenting cells according the HLA typing of MM patients and pulsed with 10 ug/ml of each of the following peptides: HLA-A2-restricted Melan-A/MART-1 (AAGIGILTV), gp100 (KTWGQYWQV, ITDQVPFSV and YLEPGPVTA), MAGE-A2 (YLQLVFGIEV and KMVELVHFL), MAGE-A3 (FLWGPRALV), NY-ESO-1 (SLLMWITQCFL), Tyr (YMDGTMSQV) SVV-1 (ELTLGEFLKL and TLPPAWQPFL), H-TERT (ILAKFLHWL and RLVDDFLLV), SOX-2 (ALSPASSRSV) or HLA-A1-restricted MAGE-A3 (EADPTGHSY) MAGE-A1 (SLFRAVITK and EVDPIGHLY), gp100 (ALLAVGATK and SLIYRRRLMK) or HLA-A3-restricted MAGE-A1 (SLFRAVITK), gp100 (SLIYRRRLMK and ALLAVGATK), or A24-restricted MAGE-A2 (EYLQLVFGI), gp100 (VYFFLPDHL), TYR (AFLPWHRLF), SVV-1 (AYACNTSTL) peptides (JPT Peptide Technologies, Berlin, Germany) (*Novellino et al., Cancer Immunol Immunother, 2004*). A panel of HLA-matched or, as control, unmatched allogeneic melanoma cell lines were used as target cells as well. The T cell recognition specifically HLA class I-restricted was determined by the inhibition of the IFN-γ release after pre-incubation of melanoma cells with the W6/32 (anti-HLA-A, B, C molecules) mAb (ATCC). T lymphocytes incubated with phytohemagglutinin (PHA) and concanavalin-A (ConA) (Sigma-Aldrich) were used as positive control for IFN-γ secretion. K562 target cells were used as control for NK-type-mediated reactivity.

Results represent averages of triplicates, subtracted of the background, with SD ≤ 10%; statistical analysis of differences between means for cytokine release assays was performed using two-tailed t-test ($p < 0.05$). PBMCs isolated from healthy donors

used for ELISPOT assay as described above represented the sample control for circulating T-cell mediated reactivity against TAAs and melanoma cell lines.

Tumor cell lines used for ELISPOT assay

The melanoma cell lines used as target cells in ELISPOT assay were: #0342, 1061, 1067, 4478D, 25368 and were established *in vitro* from human melanoma tissues at the Unit of Immuno-biotherapy of Melanoma and Solid Tumors, San Raffaele Foundation Centre, Milan, Italy. Other cell lines used were the 501 mel (a gift of Dr. Paul F. Robbins, Surgery Branch, NCI, Bethesda MD), the 15392 mel (kindly provided by Dr. Chiara Castelli, Istituto Nazionale Tumori, Milan, Italy), the Jofr-1A and Daju cell lines (kindly provided by Dr. Pierre Coulie, de Duve Institute and the Université Catholique de Louvain, Belgium), the 1061 EBV-B (HLA-A*01) established at the at the Unit of Immuno-biotherapy of Melanoma and Solid Tumors, San Raffaele Foundation Centre, and the T2 and K562 cells (ATCC). All the lines were cultured with RPMI 1640 plus 10% FBS (Lonza), 20 mM HEPES, penicillin (200 U/ml), streptomycin (200 ug/ml) and 2mM Glutamax (Invitrogen).

Legends to the Figures:

Figure 1S: Representative gating strategy for IF and cytofluorimetric analysis

PBMCs were stained according the panels described in Table 1S. $1x10^5$ events were acquired for cytofluorimetric analysis. Lymphocytes cells were gated based on physical parameters (Panel A) and sequentially gated cells were analysed on $CD3^+$ cells (Panel B), on $CD3^+CD8^+$ (Panel C), on $CD3^+CD4^+$ (Panels D and F). More subpopulations were gated on CD45RA+, CD45RO+ and on TH17 cells (data not shown). For details *see Material and Methods session*.

Figure 2S: Detection of sNKG2DLs in the serum of MM patients.

The presence of soluble NKG2DLs was measured by ELISA assay (*see Material and Methods*) in the serum at baseline (N=37; black circle), W12 (N=37; black square) and W24 (N=21; black triangle) post-treatment in MM patients undergoing IPI plus FTM treatment in the context of NIBIT-M1 study. As control the presence of soluble NKG2DLs was assessed in the serum of N=10 HD. All sera from HD resulted

negative for the presence of these molecules; only in two subjects 44 and 50 pg/ml of either ULPB-1 or ULBP-2, respectively were detected (*data not shown*). Horizontal bars represent the means (Panels A, B and C); standard deviation for normal distribution of sULBP3 has been shown in Panel C as well. Differences in means were assessed with the Student t-test ($p<0.01$).

Figure 3S: ***T cell responses against MAAs and melanoma cell lines in PBMC of melanoma patients enrolled in the NIBIT-M1 study*****.**

Ex-vivo isolated PBMCs from melanoma patients #006 (Panel A), 009 (Panel B), were used to assess their reactivity against MAA-derived peptides on HLA-A*01^{+} 1061 EBV-B (Panel A) or HLA-A*0201^{+} T2 cells (Panel B) and allogeneic HLA-matched tumor cell lines. IFN-γ release was measured by ELIspot assay and data are expressed as N. of spots/3 x 10^4 cells and are subtracted of the background of spontaneous release IFN-γ release of either T cell alone or incubated with EBV-B or T2 cells alone. The assay was performed in triplicates and their averages with SD ≤ 10 % were calculated. Each assay was performed twice and results represent averages of N. of spots/3 x 10^4 obtained from two experiments. Statistical analysis of differences between means of IFN-γ released by T cells was performed by two-tailed t-Test ($p<0.05$)

Table 1S. Panels for IF and cytofluorimetric analysis of PBMCs from MM patients and HD

Membrane staining											
CTRL -	**Tube 1**	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**	**Tube 6**	**Tube 7**	**Tube 8**	**Tube 9**	**Tube 10**	**Tube 11**
Unstained	CD134-FITC CD137-PE CD45RA-ECD CD45RO-PE-Cy7 NKG2D-APC CD4-APC-Cy7 CD8-PB CD3-V500	CD57-FITC CCR7-PE CD45RA-ECD CD45RO-PE-Cy7 CD56-APC CD4-APC-Cy7 CD8-PB CD3-V500	CD28-FITC CCR7-PE CD45RA-ECD CD45RO-PE-Cy7 CD27-APC CD4-APC-Cy7 CD8-PB CD3-V500	CD62L-FITC CCR7-PE CD45RA-ECD CD45RO-PE-Cy7 CD27-APC CD4-APC-Cy7 CD8-PB CD3-V500	CD28-FITC CD69-PE CD45RA-ECD CD45RO-PE-Cy7 NKG2D-APC CD4-APC-Cy7 CD8-PB CD3-V500	CD152-FITC CD278-PE CD45RA-ECD CD45RO-PE-Cy7 NKG2D-APC CD4-APC-Cy7 CD8-PB CD3-V500	CD152-FITC CD272-PE CD45RA-ECD CD45RO-PE-Cy7 CD279-APC CD4-APC-Cy7 CD8-PB CD3-V500	IL23R-FITC CD183-PE CD196-PercP-Cy5.5 CD45RO-PE-Cy7 CD195-APC CD4-APC-Cy7 CD8-PB CD3-V500	CD152-FITC CD183-PE CD196-PercP-Cy5.5 CD45RO-PE-Cy7 CD161-APC CD4-APC-Cy7 CD8-PB CD3-V500	IL-12 Rβ2-FITC CD278-PE CD196-PercP-Cy5.5 CD45RO-PE-Cy7 CD279-APC CD4-APC-Cy7 CD8-PB CD3-V500	CD19-FITC CD272-PE CD22-APC CD4-APC-Cy7 CD8-PB CD3-V500

Intracellular staining											
CTRL -	**Tube 12**										
Unstained	CD4-FITC IL-17-Alexa Fluor 700 CD8-PB CD3-V500										

CTRL-: Control sample for setting of physical parameters for cytofluorimetric analysis; it was represented by unstained PBMCs. The mAbs were combined in each single tube as indicated in the Table; for details *see Material and Methods session*.

Table 2S. MAA and/or tumor reactivity by PBMCs from melanoma patients undergoing *IPI* + FTM treatment

Antigen reactivity	**Baseline** [a]	**W12** [b]		
		Pre-existing [c]	**Augmented responses** [d]	**New** [e]
MAGE- A1	**2**	1	0	**4**
MAGE-A2	**1**	0	0	**2**
MAGE-A3	0	0	0	0
NY-ESO-1	0	0	0	**4**
MART-1	**2**	1	0	**3**
Gp100	**3**	2	**1**	**3**
Tyros.	**9**	7	**4**	**1**
h-TERT	**5**	1	**4**	0
SVV-1	0	0	0	**1**
SOX-2	0	0	0	0
Tumor reactivity	**Baseline** [a]	**W12** [b]		
		Pre-existing [c]	**Augmented responses** [d]	**New** [e]
HLA-A1	0			
HLA-A2	**5**	2	**3**	**2**
HLA-A3	**4**	3	0	0

PBMCs from MM patients (N=23) at baseline (a) and W12 (b) post-IPI plus FTM treatment were used to assess by IFN-γ release assay (ELISPOT) the reactivity against MAAs and HLA-matched allogeneic tumor cells.

[a] N. of patients with T cell-mediated reactivity against the indicated MAA or HLA-matched melanoma cell lines;

[c] N. of patients with T cell-mediated reactivity against the indicated MAA or HLA-matched melanoma cell lines that were already detected at baseline;

[d] N. of patients with T cell responses against the indicated MAA or HLA-matched melanoma cell lines augmented (at least twice the mean of the N. of spot/3 x 10^4 cells) as compared with baseline;

[e] Newly induced post-treatment T cell responses against the indicated MAA or HLA-matched melanoma cell lines.

Figure 1S. Representative gating strategy of IF and cytofluorimetric analysis of PBMC

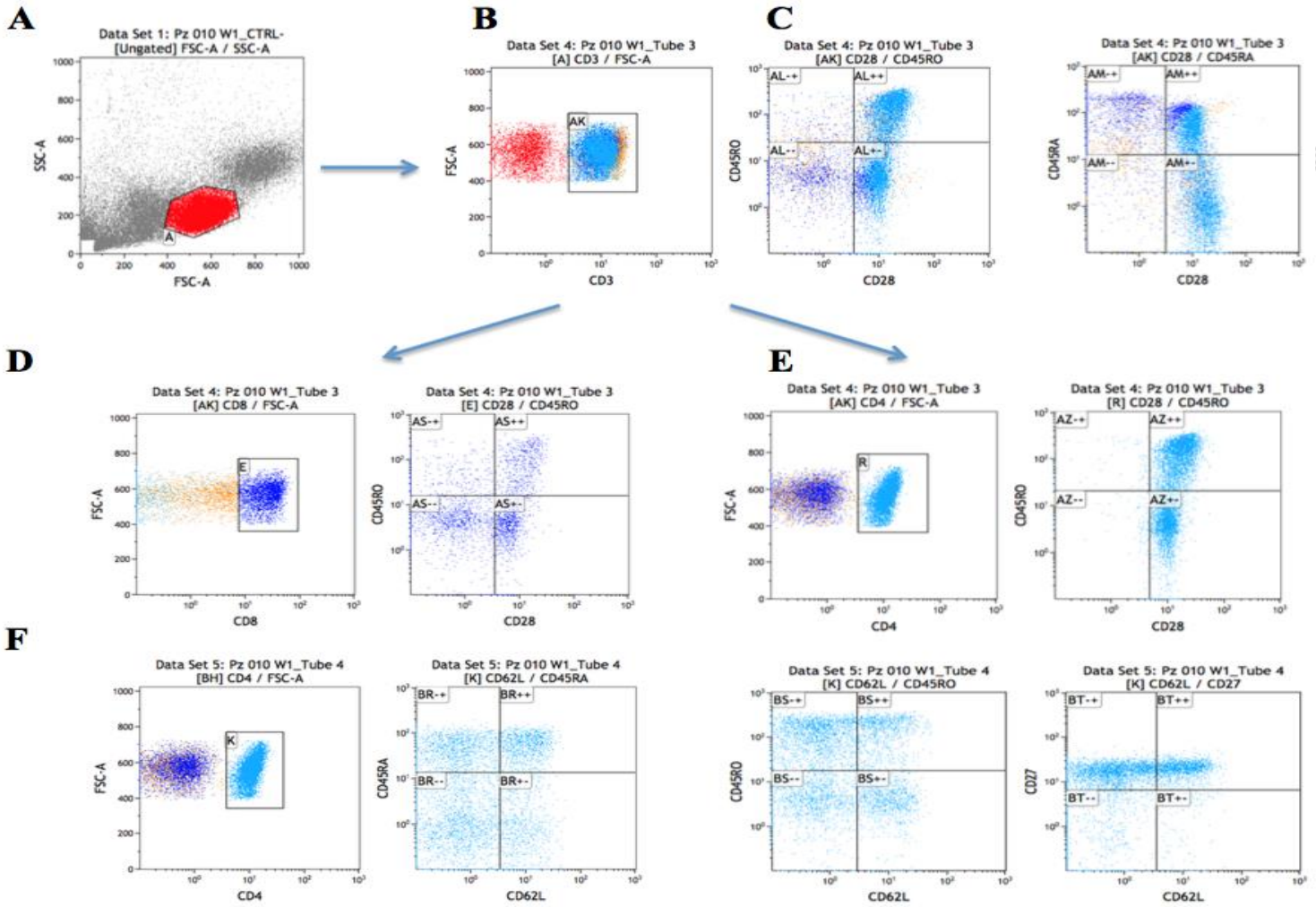


Figure 2S

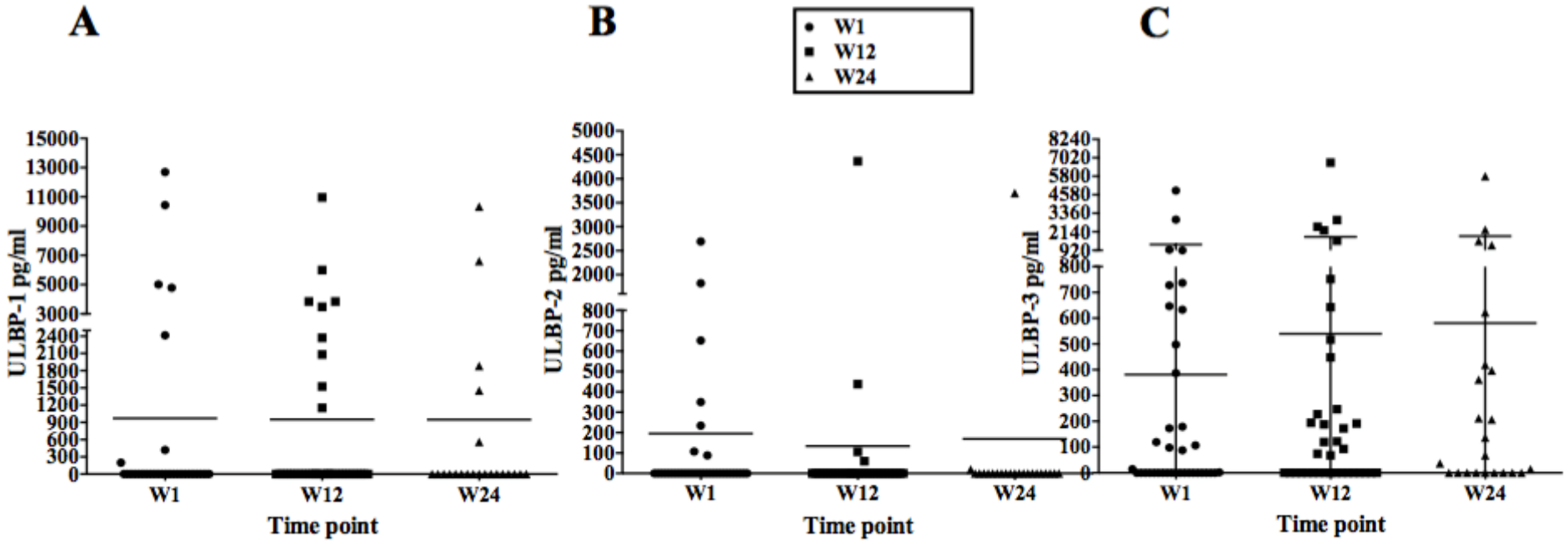
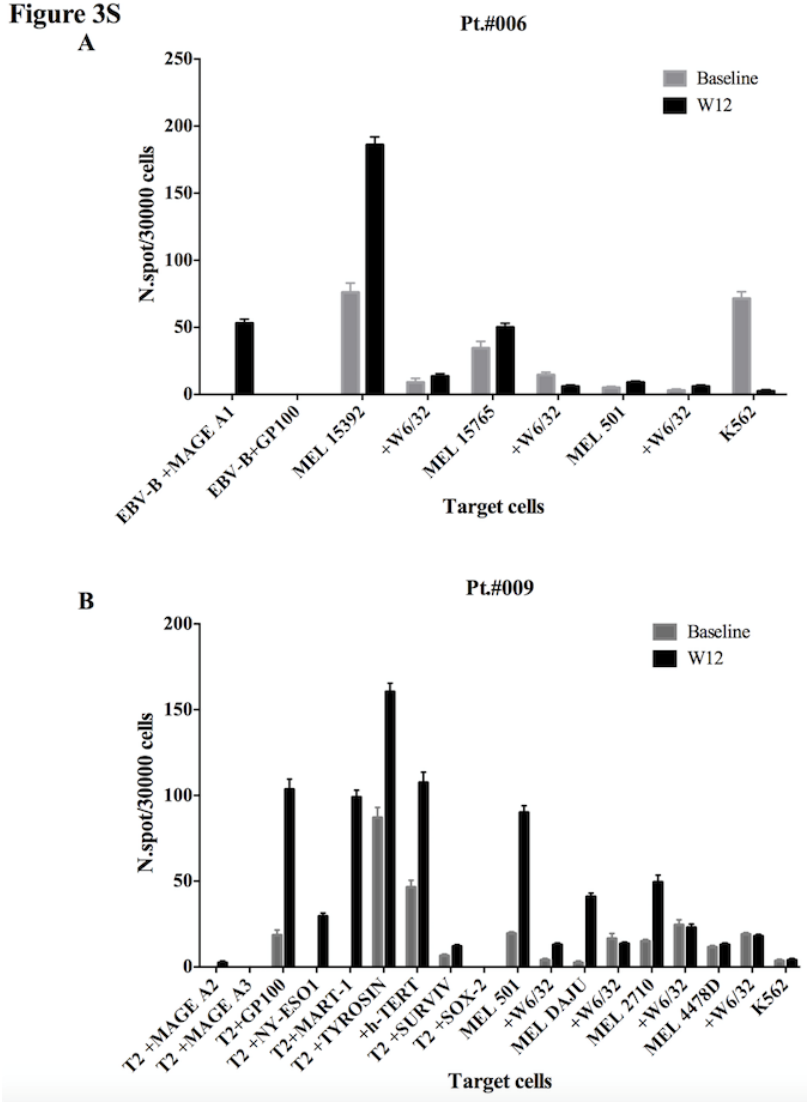

A
B
C
W1
W12
W24
ULBP-1 pg/ml
ULBP-2 pg/ml
ULBP-3 pg/ml
Time point
Time point
Time point

Figure 3S


A
Pt.#006
Baseline
W12
N.spot/30000 cells
250
200
150
100
50
0
EBV-B +MAGE A1
EBV-B+GP100
MEL 15392
+W6/32
MEL 15765
+W6/32
MEL 501
+W6/32
K562
Target cells
B
Pt.#009
Baseline
W12
N.spot/30000 cells
200
150
100
50
0
T2 +MAGE A2
T2 +MAGE A3
T2+GP100
T2 +NY-ESO1
T2+MART-1
T2 +TYROSIN
+h-TERT
T2 +SURVIV
T2 +SOX-2
MEL 501
+W6/32
MEL DAJU
+W6/32
MEL 2710
+W6/32
MEL 4478D
+W6/32
K562
Target cells