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ORIGINAL RESEARCH

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Antigen-specificity and DTIC before peptide-vaccination differently shape immune-checkpoint expression pattern, anti-tumor functionality and TCR repertoire in melanoma patients

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ABSTRACT

We have recently described that DNA-damage inducing drug DTIC, administered before peptide (Melan-A and gp100)-vaccination, improves anti-tumor CD8$^+$ Melan-A-specific T-cell functionality, enlarges the Melan-A$^+$ TCR repertoire and impacts the overall survival of melanoma patients. To identify whether the two Ags employed in the vaccination differently shape the anti-tumor response, herein we have carried out a detailed analysis of phenotype, anti-tumor functionality and TCR repertoire in treatment-driven gp100-specific CD8$^+$ T cells, in the same patients previously analyzed for Melan-A. We found that T-cell clones isolated from patients treated with vaccination alone possessed an Early/intermediate differentiated phenotype, whereas T cells isolated after DTIC plus vaccination were late-differentiated. Sequencing analysis of the TCRBV chains of 29 treatment-driven gp100-specific CD8$^+$ T-cell clones revealed an oligoclonal TCR repertoire irrespective of the treatment schedule. The high anti-tumor activity observed in T cells isolated after chemo-immunotherapy was associated with low PD-1 expression. Differently, T-cell clones isolated after peptide-vaccination alone expressed a high level of PD-1, along with LAG-3 and TIM-3, and were neither tumor-reactive nor polyfunctional. Blockade of PD-1 reversed gp100-specific CD8$^+$ T-cell dysfunctionality, confirming the direct role of this co-inhibitory molecule in suppressing anti-tumor activity, differently from what we have previously observed for Melan-A$^+$CD8$^+$ T cells, expressing PD-1 but highly functional. These findings indicate that the functional advantage induced by combined chemo-immunotherapy is determined by the tumor antigen nature, T-cell immune-checkpoints phenotype, TCR repertoire diversity and anti-tumor T-cell quality and highlights the importance of integrating these parameters to develop effective immunotherapeutic strategies.

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Introduction

The primary goal of cancer immunotherapy is the generation of a strong and persistent T-cell mediated anti-tumor immune response, able to control tumor progression. A number of therapeutic peptide-vaccination protocols have been developed to induce CD8$^+$ T cell responses against the tumor by administering antigenic peptides expressed by cancer cells combined with adjuvants.[1,2] Hence, the need to identify relevant peptides eliciting efficient T-cell responses and specific CD8$^+$ T-cell receptor (TCR) repertoire, able to recognize and eliminate tumor cells.[1,3] A critical determinant is to study the TCR-MHC complex in terms of affinity/avidity, and its impact on T-cell differentiation, survival, persistence as well as tumor reactivity.

A full T-cell activation and efficient anti-tumor functionality depend on the balance between signals from both positive and negative co-stimulatory molecules which regulate the distinctive phases and the quality of the T-cell response.[4,5] The co-stimulatory molecule CD28 plays a critical role in supporting TCR-mediated naïve and memory T-cell activation, clonal expansion and acquisition of effector functions,[6,7] while CD27 controls TCR activation and promotes survival of activated T cells.[8,9] The loss of both CD28 and CD27 molecules defines a late differentiation status of T cells.[10,11] On the other hand, inhibitory receptors, including Lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin domain-3 (TIM-3) and Programmed death-1 (PD-1) have been strongly associated with "T-cell exhaustion".[12]

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However, PD-1 expression may also reflect a particular differentiation and/or activation status of T cells. In fact, besides its inhibitory effect on T-cell function, mainly exerted by dephosphorylating CD28,[14] PD-1 has recently been shown to be highly expressed in the effector phase of T cells, particularly in melanoma-reactive and clonally expanded $CD8^+$ T-cells.[15,16]

The majority of cancer vaccination trials have only provided partial results in the induction of an efficient anti-tumor cytotoxic T lymphocyte (CTL) response, due to the poor immunogenicity of antigenic peptides when administered alone,[17] suggesting the need for more powerful vaccines also in combination with standard chemotherapy[18-20] or with immune checkpoint inhibitory antibodies.[21-23] We have recently reported that patients receiving dacarbazine (DTIC) one day before peptide (Melan-A and gp100)-vaccination plus interferon (IFN)-α show a progressive enhancement of the TCR repertoire diversity of Melan-A-specific $CD8^+$ T cells, accompanied by the maintenance of highly-avid and beneficial anti-tumor activity, as compared with cells isolated from patients receiving vaccination alone.[18] These highly anti-tumor reactive and multifunctional $CD8^+$ T cells were not impaired by the presence of a high level of PD-1 and showed an AKT activation pathway sustained by the inducible co-stimulator (ICOS) molecule.[16] In contrast, patients treated with peptide vaccination alone showed a reduction of Melan-A^+ TCR repertoire diversity accompanied by a reduction of tumor lytic activity.

To identify $gp100^+$ TCR clonotypes able to provide an effective anti-tumor immune response we analyzed the TCR repertoire and the functional activity of gp100-specific $CD8^+$ T cell clones isolated from melanoma patients after the treatments and evaluated the differences in the response against the two different antigens (Ags) employed in our vaccination protocol. The comparison between gp100 and Melan-A specific T-cell response in the same patient revealed that, differently from that observed for Melan-A,[18] an oligoclonal $gp100^+$ TCR repertoire was identified independently of the treatment. On the other hand, the two treatments differently affected immune-checkpoint phenotype and functionality. The gp100-specific $CD8^+$ T cells isolated after vaccination alone were unable to kill tumor cells, showed low anti-tumor polyfunctionality and expressed the highest levels of inhibitory molecules, with PD-1 involved in the poor anti-tumor functionality of these cells, whereas those isolated after chemo-immunotherapy were highly functional and PD-1 negative.

Our data, again, indicate the ability of combined chemo-immunotherapy to induce a highly effective anti-melanoma immune response and highlight how two diverse Ags can differently shape a specific T-cell response in the same patient, in terms of immune-checkpoint pattern expression and TCR repertoire. This observation provides important information to design new therapy combinations able to improve the Ag-specific anti-tumor response.

Results

Oligoclonality of gp100-specific $CD8^+$ T cells isolated from melanoma patients treated with peptide-vaccination alone or in combination with DTIC

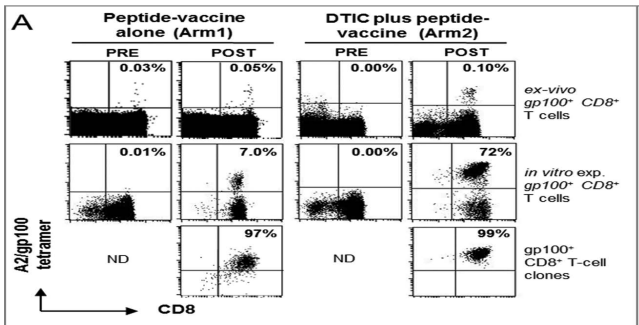
We have recently reported that a combination of DTIC injected one day before vaccination with Melan-A and gp100 peptides plus IFN-α (Arm2) improves the anti-tumor lytic activity of Melan-A-specific $CD8^+$ T cells, as compared with peptide-vaccination alone (Arm1), and impacts the overall survival of melanoma patients.[16,18] To identify functional differences in the response against the two different Ags employed in the vaccination protocol, we analyzed the endogenous and therapy-induced anti-gp100 T-cell response in patients treated with both treatment schedules, and then generated a panel of specific $gp100^+$ $CD8^+$ CTL clones. Representative *ex-vivo* (upper panel) and *in-vitro* shortly expanded (lower panel) gp100/tetramer-staining dot plots are shown in Fig. 1A, while Fig. 1B summarizes the *ex vivo* endogenous response, the different *in vitro* expansion potential of gp100 specific $CD8^+$ T cells and all the $gp100^+$ T-cell clones isolated after the two treatment schedules.

Differently from what observed for Melan-A,[19] the endogenous anti-gp100 response (PRE) was very low or undetectable, hampering the generation of gp100-specific $CD8^+$ T-cell clones (Fig. 1B). In contrast, after both treatments we were able to isolate a large number of gp100-tetramer-positive $CD8^+$ T-cell clones from three patients, who showed specific expansion in both *ex-vivo* and *in-vitro* short-term Ag-sensitized $CD8^+$ T cells (Fig. 1A and B).

We previously demonstrated that the administration of combined chemo-immunotherapy is associated with the rise of Melan-A-specific $CD8^+$ T-cell clones characterized by a wide TCR repertoire and highly polyfunctional anti-tumor activity.[16,18] To analyze whether the different treatments contributed to shaping the Ag-specific TCR repertoire in a peptide-dependent manner, we analyzed the T-Cell Receptor Beta Variable (TRBV) of 37 gp100-specific $CD8^+$ T-cell clones elicited by the two different vaccination protocols.

From the analysis of complementarity-determining region (CDR3) sequences we identified nine different clonotypes from the 29 sequences with in frame rearrangements of TRBV, TRBD, TRBJ and TRBC segments (Fig. 1C and Table S1). When we evaluated each patient we found that treatment-driven gp100-specific TCRBV showed high similarities in the amino acid sequence, while no similarities were shared among the patients (Fig. 1C). Moreover, gp100-specific TCRs expressed an oligoclonal repertoire irrespective of the treatments (Arm1, Arm2). In detail, as shown in Fig. 1C in patient 08, treated with vaccination alone, clonotype 1 was present in 6 out of 9 $CD8^+$ T-cell clones sequenced (66.6%). The clonotype/clone ratio, that we have previously described as an index of TCR diversity,[18] was 0.33. Among the $gp100^+$ $CD8^+$ T cells isolated after combined chemo-immunotherapy, 7 out of 9 clones from patient 15 expressed the same clonotype (ID 4, 77.7%) (clonotype/clone ratio 0.22). In patient 38, we identified four clonotypes, with clonotype 6 expressed in 6 out of the 11 clones isolated (54.5%) (clonotype/clone ratio 0.36). Moreover, the CDR3 length analysis showed that, in each of the three patients, the clonotype with the highest frequency is also characterized by a longer CDR3 sequence (Fig. 1C).

These findings indicate that in this clinical setting the gp100-peptide-vaccination elicits an oligoclonal TCRBV repertoire not diversified by the combined treatment with DTIC.



B

Patient ID	Arm*	Time**	gp100+ CD8+ *ex-vivo* (%) ***	gp100+ CD8+ *in vitro* exp. (%)***	Proliferating clones (No)	gp100+ CD8+ clones (No)***	Sequenced clones (No)
Pt 08	1	PRE	0.03	0.01	ND	ND	9
		POST	0.05	7.0	165	10 (6%)	
Pt 15	2	PRE	0.01	0.01	ND	ND	9
		POST	0.13	90.0	16	16 (100%)	
Pt 38	2	PRE	0.00	0.00	ND	ND	11
		POST	0.10	72.0	58	43 (74%)	

C

	Pt	ID	CDR3-IMGT (105 106 107 108 109 110 111 111.1 111.2 112.2 112.1 112 113 114 115 116 117)	CDR3 (aa)	BV	BJ	BD	BC	clones	Frequency	(%)	clonotype/ clone ratio
Arm 1	08	1	A S S L R P G T G G A Y N S P L H	17	4-1	1-6	1	1	7, 63, 69, 81, 98, 117	6/9	(66.6)	
		2	A S S L M G R . . . . A N Q P Q H	13	7-8	1-5	1	1	126, 168	2/9	(22.2)	0.33
		3	A S S L M Q . . . . . G T E A F	11	27	1-1	1	1	24	1/9	(11.1)	
Arm 2	15	4	A S T L W G S . . . R G D T E A F	14	6-2	1-1	1	1	2, 3, 4, 6, 11, 19, 24	7/9	(77.7)	
		5	A S S F Q G . . . . . P Y G Y T	11	28	1-2	1	1	13, 16	2/9	(22.2)	0.22
	38	6	A S S L Y P R G . . G G F Y G Y T	15	27	1-2	1	1	1, 4, 12, 35, 81, 105	6/11	(54.5)	
		7	A S S L G R . . . . . G N E Q Y	11	27	2-7	2	2	39, 43	2/11	(18.1)	0.36
		8	A S S L G P S . . . G R V N E Q F	14	12-3	2-1	2	2	25, 78	2/11	(18.1)	
		9	A S S N L A G . . . . G P Y E Q Y	13	9	2-7	2	2	49	1/11	(9.0)	

Figure 1. Generation and sequencing of gp100-specific CD8$^+$ T-cell clones. (A). Representative example of HLA-A2/gp100 tetramer staining in endogenous *ex-vivo* CD8 T cells (upper), short-term Ag-sensitized CD8$^+$ T cells (middle), and T-cell clones (lower), in Arm1 (Pt08) and Arm2 (Pt38) patients. ND, not done. (B). *Ex-vivo* immune monitoring and generation of gp100$^+$CD8$^+$ T-cell lines and clones. * Arm1, peptide-vaccine alone; Arm2, DTIC plus peptide-vaccine.** Time of immune monitoring and T-cell cloning. *** Percentage of gp100-positive CD8$^+$ T cells as detected by tetramer staining; ND, not done. (C). Amino acid sequences of TCRBV of treatment-driven gp100-specific T-cell clonotypes. The sequences were analyzed, numbered and classified according to the IMGT indications (IGMT Repertoire http://igmt.cines.fr). The ratio between the number of identified clonotypes and the total number of clones sequenced is indicated for each patient, which represents an index of TCR diversity.[18] ID, clonotype sequence identification; Pt, patients identification.

Chemo-immunotherapy induces a late differentiated phenotype, while vaccination alone maintains early differentiated clonotypes in CD8$^+$ gp100-specific T cells

To evaluate the differentiation status of the identified gp100-specific clonotypes we carried out an in depth analysis of the T-cell phenotype by flow cytometry (Fig. S1).[11,24]

We found that 46 CD8$^+$ gp100-specific T-cell clones showed an effector memory CCR7$^-$CD45RA$^-$ phenotype irrespective of treatment (Table 1 and Fig. S1B), in accordance with Geginat et al, showing how *in vitro* TCR-stimulated cells acquire a CCR7$^-$CD45RA$^-$ phenotype.[25] On the other hand, the analysis of CD28 and CD27 receptors indicated that the clones display a different degree of differentiation depending on the treatment (Table 1). In particular, T cells isolated from patients treated with vaccination alone showed a less differentiated phenotype in terms of CD28 expression (Arm1, mean ± SD 56% ± 27%), as compared with cells isolated from patients treated with chemo-immunotherapy (Arm2, mean ± SD 6% ± 10%; $P < 0.0001$). The intermediate differentiated profile of gp100$^+$ T-cell clonotypes derived from vaccination alone was also supported by the expression analysis of C-X-C chemokine receptor type 5 (CXCR5), a marker recently associated with memory stem cell-like CD8$^+$ T cells,[26,27] significantly higher in Arm1 (mean ± SD 43% ± 6.9%) as compared with Arm2 (mean ± SD 34% ± 3.3%, $P = 0.03$), either alone (Table 1) or in co-expression with CD28 ($P = 0.006$, data not shown). The analysis of the expression of CD56 and Natural Killer Group 2D (NKG2D), markers for activation and effector functionality, revealed no difference for CD56 but a heterogeneous expression for NKG2D (Table 1).

Chemo-immunotherapy promotes the expansion of tumor-reactive and highly avid TCR clonotypes, while vaccination alone induces gp100$^+$ T cells ineffective against the tumor

To identify highly avid and tumor-reactive gp100-specifc clonotypes, we characterized the anti-tumor functionality of T-cell clones by ^{51}Cr-release cytotoxicity assays, using melanoma cell lines negative or positive for gp100 expression. Only clones isolated after chemo-immunotherapy (Arm2) showed high tumor lytic activity, while clones isolated after peptide-vaccination alone (Arm1) were unable to lyse melanoma cells ($P < 0.0001$; Fig. 2A, left panel). It is noteworthy that the blockade of MHC-I with W6-32 mAb inhibited tumor lysis by 50%, demonstrating the MHC-restricted Ag-specificity of the anti-tumor lytic activity of these clones (Fig. 2A, middle panel). To analyze whether the lack of anti-tumor response observed in Arm1 patients was mediated by a deficiency in the recognition of wild type gp100$_{209–217}$ Ag (wt), we tested the T-cell clones against melanoma cells pulsed with the analogue gp100$_{209–217}$ 210M peptide (210M), without any significant improvement of tumor lysis (Fig. 2A, middle panel). The functional differences observed in clones isolated after the different treatments could not be ascribed to an intrinsic dysfunctionality of CD8$^+$ T cells, since all gp100-specific T-cell clones analyzed were able to recognize the analogue gp100$_{209–217}$ 210M peptide presented by T2 target cells with high efficiency (Fig. 2A, right panel).

We then investigated the T-cell polyfunctional pattern in terms of co-production of Granzyme-B (GrB), tumor necrosis factor (TNF)-α and IFN-γ, in response to the melanoma cell line Mel3 (HLA-A2$^+$ gp100$^+$) (Fig. S1C). The analysis of the

Table 1. Characteristics of gp 100-specific T-cell clones.

					Phenotype						
Patient ID	Arm	Time*	Clone ID	Clonotype ID**	NKG2D (%)***	CD56 (%)***	CCR7 (%)***	CD45RA (%)***	CD28 (%)***	CD27 (%)***	CXCR5 (%)***
08	1	POST	7	1	10	15	nd	nd	83	nd	nd
			63	1	67	33	nd	4	85	nd	34
			69	1	74	30	nd	nd	50	30	nd
			81	1	30	4	0	0	65	10	51
			98	1	89	37	nd	nd	8	6	44
			117	1	12	26	0	5	70	20	42
			126	2	0	3	0	10	70	5	nd
			168	2	0	2	0	5	70	5	42
			24	3	21	2	0	7	43	9	43
			128	nd	27	27	nd	nd	12	nd	nd
			mean ± SD		33 ± 32	18 ± 14	0	5 ± 3	56 ± 27	12 ± 9	43 ± 5.4
15	2	POST	2	4	57	10	nd	nd	5	55	35
			3	4	25	28	nd	nd	0	nd	nd
			4	4	35	35	0	5	0	2	nd
			6	4	34	29	nd	nd	8	60	39
			11	4	0	87	nd	2	0	0	nd
			19	4	0	15	nd	nd	0	5	31
			24	4	0	26	0	0	0	nd	40
			13	5	0	7	0	5	30	0	nd
			16	5	0	3	0	15	32	0	nd
			15	nd	3	18	0	2	2	5	35
			17	nd	0	22	nd	nd	0	nd	nd
			mean ± SD		14 ± 20	33 ± 23	0	4.8 ± 5	7 ± 13	16 ± 26	35 ± 3.6
38	2	POST	1	6	nd	nd	nd	nd	nd	nd	nd
			4	6	75	30	0	0	20	61	30
			12	6	88	63	0	12	3	27	34
			35	6	0	5	nd	nd	0	nd	nd
			81	6	50	25	0	0	4	21	48
			105	6	90	8	nd	nd	6	45	nd
			39	7	5	33	nd	5	0	0	nd
			43	7	80	33	0	4	0	0	nd
			25	8	18	0	0	0	17	20	36
			78	8	7	0	nd	nd	0	nd	28
			49	9	0	70	nd	15	4	0	nd
			63	nd	70	53	nd	10	0	nd	nd
			65	nd	90	75	nd	8	0	nd	nd
			86	nd	90	31	nd	nd	0	nd	nd
			70	nd	0	60	0	3	0	nd	nd
			88	nd	80	0	0	0	0	nd	nd
			7	nd	50	95	nd	nd	0	nd	nd
			40	nd	30	17	nd	10	0	nd	nd
			41	nd	15	37	nd	8	0	nd	nd
			55	nd	0	42	0	0	0	nd	nd
			28	nd	0	15	0	7	0	nd	nd
			21	nd	0	10	nd	nd	0	nd	nd
			58	nd	0	10	nd	8	0	nd	nd
			45	nd	23	44	nd	nd	0	nd	nd
			33	nd	80	33	nd	nd	0	nd	nd
			mean ± SD		39 ± 37	33 ± 26	0	6 ± 5	2 ± 5	22 ± 24	35 ± 7.9
		Arm2	mean ± SD		32 ± 35	29 ± 25	0	5.4 ± 5	6 ± 10	19 ± 23	35 ± 5.8
			*p - value***		*0.91*	*0.18*	*na*	*0.9*	*<0.0001*	*0.34*	*0.03*

Time of T-cell cloning; **Identification number (ID) of each clonotype identified by TCR β-chain sequencing; ***Values represent the mean of three-five independent experiments; ****Arm1 versus Arm2, unpaired Student's t-test; nd, not determined; na, not applicable.

single and simultaneous molecule expression evidenced that gp100-specific $CD8^+$ T-cell clones isolated after vaccination alone (Arm1) showed low levels of GrB, TNF-α and IFN-γ Fig. 2B), consistent with their low anti-tumor lytic activity. Notably, T cells isolated after combined chemo-immunotherapy (Arm2) showed significantly higher levels of GrB and IFN- , and not of TNF-α, as compared with the levels observed after vaccination alone (Fig. 2B). When we analyzed the co-expression pattern, we found a significant increase in the simultaneous GrB and IFN-γ production, and a trend towards statistical significance when all the three molecules were analyzed (Fig. 2B, right panel, $P = 0.07$).

We then evaluated the fine specificity of Ag recognition (Fig. 2C for representative clones), towards either the wild type (wt) or the analogue (210M) gp100 peptide, the latter employed in our vaccine formulation. Functional avidity analysis (as defined by the concentration of peptide required to obtain 50% maximal lysis, EC_{50}) showed that most clones isolated from patients treated with combined chemo-immunotherapy (Arm2) showed a 'high' and 'very high' avidity (as scored in Fig. 2D) towards both the analogue (~80%) and the wild type (100%) gp100 peptides (Fig. 2D). Differently, all clones isolated after vaccination alone (Arm1) responded to the analogue gp100 peptide with 'intermediate'/'high' avidity. Fifty-seven

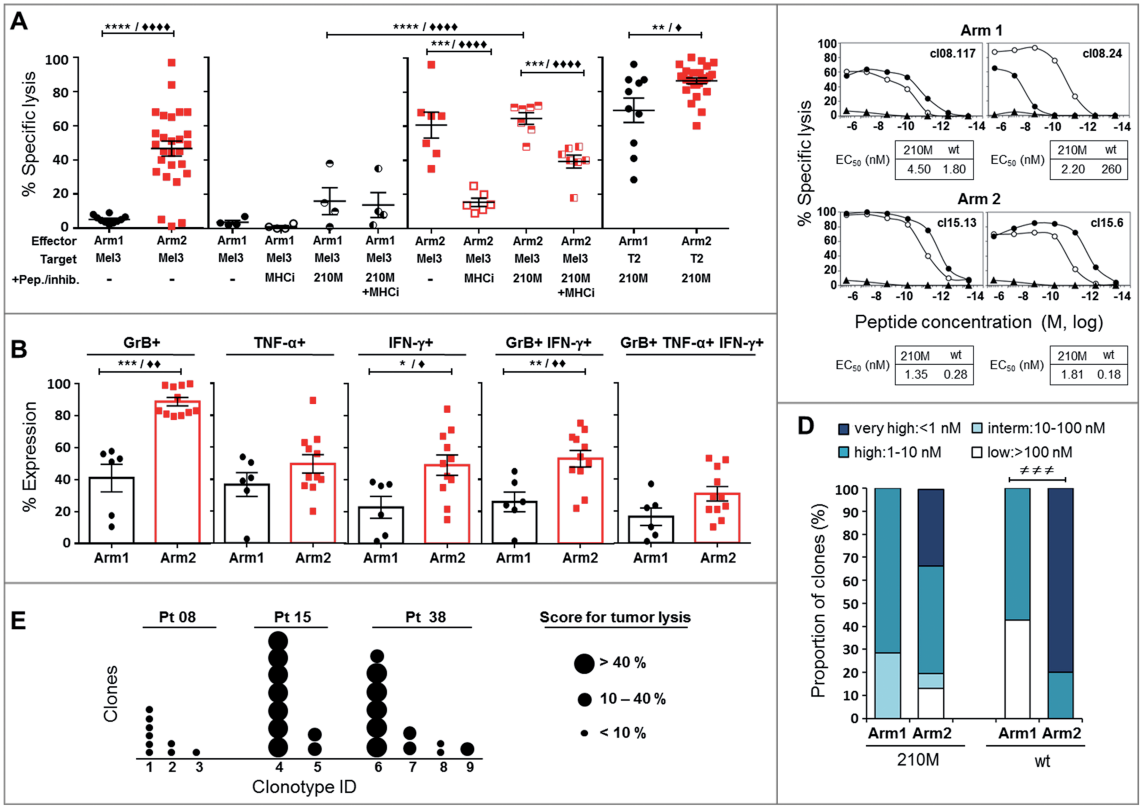


Figure 2. Chemo-immunotherapy elicits tumor-reactive and highly avid gp100-specific TCR clonotypes. (A). Percentage of specific lytic activity of 37 gp100-specific T-cell clones isolated from patients of Arms1 and 2. Each dot represents the mean value from three independent experiments performed with a single T-cell clone. Lytic activity was analyzed against $A2^+/gp100^+$ melanoma cell line (Mel3), pulsed or not with analogue $gp100_{209–217}$ 210M peptide (210M), with or without MHC-I inhibition (W6-32 mAb), and against T2 cells pulsed with gp100 210M peptide (E:T ratio of 20:1). The percentage of specific lysis was obtained by subtracting the nonspecific lysis value (<5%). The mean ± SEM of each sample group is shown. (B). Percentage of single or simultaneous co-production of GrB, TNF-α and IFN-γ, as measured by multicolour ICS in 17 gp100-specific T-cell clones stimulated with $A2^+/gp100^+$ melanoma cell line. Each dot represents the mean value from three independent experiments performed with a single T-cell clone. The percentage of molecule production against melanoma cell lines Mel1 ($HLA\text{-}A2^-/gp100^+$), or Mel2 ($HLA\text{-}A2^+/gp100$) was below 5% (not shown). $^{*/\blacklozenge}$ $P \leq 0.05$, $^{**/\blacklozenge\blacklozenge}$ $P \leq 0.01$, $^{***/\blacklozenge\blacklozenge\blacklozenge}$ $P \leq 0.001$, $^{****/\blacklozenge\blacklozenge\blacklozenge\blacklozenge}$ $P \leq 0.0001$, Mann-Whitney two-sample test (*) and two-tail Student's test ($^\blacklozenge$), respectively. In the simultaneous molecule co-expression a trend towards statistical significance was observed ($P = 0.07$, Student's test). (C). Fine Ag specificity analysis of two representative clones from each Arm, measured by cytotoxicity assay using T2 target cells pulsed with decreasing concentrations of wild type $gp100_{209–217}$ (wt, ●), analogue gp100 210M (210M, ◯) or irrelevant Melan-A (▲) peptides. Avidity of T cells, defined as the concentration of peptide required for half-maximal lysis (EC_{50}), is shown. (D). $CD8^+$ T-cell clones were grouped in four degrees of avidity strength, from low to very high, and the relative percentage is represented by the sections inside the bars. $^{\neq\neq\neq}$ $P \leq 0.001$, Pearson chi-square test. (E). Frequency, lytic activity and TCR diversity of gp100-specific $CD8^+$ T-cell clonotypes. The identification (ID) number of each clonotype is reported in the horizontal axis. The anti-tumor lytic activity of each T-cell clone against $A2^+/gp100^+$ melanoma cell line (E:T ratio of 20:1) is reported in three scores.

percent of Arm1 clones were able to recognize the wild type peptide with 'high' avidity, although unable to lyse the tumor cells. We never found clones from Arm1 able to recognize either of the peptides with 'very high' avidity (EC_{50} < 1nM) (Fig. 2D).

When we compared the anti-tumor lytic activity of 29 $gp100^+$ T-cell clones, scored and represented by the different size of the circle illustrated, with their TCR repertoire diversity (Fig. 2E), we found that all patients showed a narrow TCR repertoire not related to the extent of lytic activity. In particular, patient 08 from Arm1 showed a restricted gp100-specific TCR repertoire and low lytic activity (clonotype/clone ratio 0.33 and tumor lysis < 10%), similarly to what we have observed for Arm1 Melan-A-specific response (Fig. 1C, 2E and [18]). In patients from Arm2 we observed a narrow gp100-specific TCR repertoire, nonetheless associated with a high functional profile in terms of anti-tumor lytic activity (Fig. 2A) and polyfunctionality (Fig. 2B).

These data suggest that gp100 peptide vaccination, when combined with DTIC, determines high tumor avidity, but this does not correspond to increased diversity of the Ag-speci TCR repertoire respect to vaccination alone.

Peptide-vaccination alone induces a high level of PD-1 expression in gp100 specific $CD8^+$ T cells, related to a reduced anti-tumor activity

While most gp100-specific T-cell clones isolated from patients treated with vaccination alone recognized the cognate Ag when presented by PD-L1-negative ([28] and data not shown) T2 cells (Fig. 2A right panel and Fig. 2D), they showed a poor Ag recognition when gp100 was expressed in the context of PD-L1-positive melanoma tumor cells (Fig. 2A left panel and 2B). Thus, we analyzed whether the functional impairment of these clones was associated with an increased expression of checkpoint inhibitory receptors (Fig. S1D). The expression profile of LAG-3, TIM-3 and PD-1 of $gp100^+CD8^+$ T-cells, isolated from both Arms of treatments, showed that in unstimulated T cells the LAG-3 molecule was undetectable or weakly expressed in all clones, while TIM-3 was well expressed, although to a

higher extent in cells isolated after vaccination alone (Fig. 3A, upper panel, and Fig. S1D). Of note, we found the highest expression of PD-1 on gp100$^+$ CD8$^+$ T cells isolated after treatment with vaccination alone and a low expression in clones isolated after combined chemo-immunotherapy ($P < 0.0001$) Fig. 3A, upper panel). Interestingly, in unstimulated cells the simultaneous presence of LAG-3, TIM-3 and PD-1 was found only in gp100$^+$ T cells isolated after vaccination alone (Fig. 3A, upper panel). When T cells were activated with anti-CD3 mAb, an increase in LAG-3 and PD-1 molecules was observed in all the clones analyzed, but again cells isolated after vaccination alone showed the highest presence of LAG-3 and PD-1, either alone or co-expressed with the other inhibitory molecules Fig. 3A, bottom panel).

Notably, the analysis of all the clones showed a statistically significant inverse correlation between PD-1 expression and low polyfunctionality in gp100-specific CD8$^+$ T cells (Fig. 3B).

The direct role of PD-1/PD-L1 engagement in the poor polyfunctional profile displayed by gp100-specific CD8$^+$ T cells was then evaluated. PD-1 blockade with a PD-1 specific mAb, before exposure to PD-L1-positive melanoma cells, induced a 58% increase of GrB, IFN-γ and TNF-α co-production in the representative clone isolated after vaccination alone (Fig. 3C).

This observation indicates a role of PD-1/PDL-1 pathway in the poor functionality of gp100-specific T-cells isolated after vaccination alone.

Comparison between phenotype, functionality and TCR diversity of Melan-A- and gp100-specific CD8$^+$ T cells in the same patients

Taking into account our previous results on Melan-A clonotypic composition and anti-tumor activity in melanoma patients,[18] and the observations described herein about the gp100-driven response, we reasoned that it would be important to compare the differences between the two Ags in shaping T-cell activity in the same patient. We evaluated the Ag-specific TCR repertoire along with selected immunological parameters including CD28 co-stimulatory and PD-1 inhibitory receptor expression and the Melan-A- and gp100-specific CD8$^+$ T-cell anti-tumor functionality (Fig. 4A and B). This comparative analysis was performed in the three patients from whom we were able to isolate T-cell clones specific for both the Ags employed in the vaccine formulation. In particular we analyzed one patient treated with peptide-vaccination alone (Pt08) and two patients with chemo-immunotherapy (Pt15 and Pt38). Analysis of CD28 and PD-1 expression was performed in

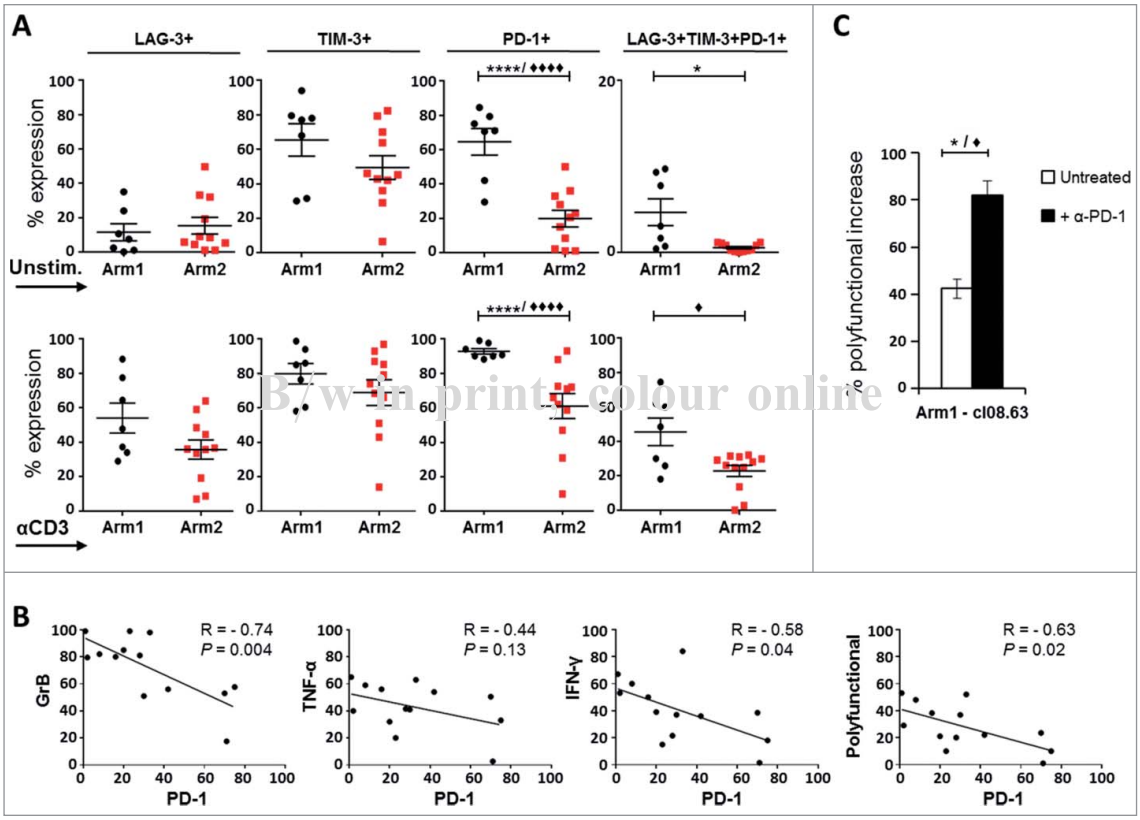


Figure 3. gp100-specific CD8$^+$ T-cell clones isolated after peptide-vaccination express a high level of the PD-1 inhibitory molecule. (A). Percentage of single or simultaneous inhibitory receptor expression in CD8$^+$ T-cell clones isolated at the end of both treatments, with (lower panel) or without (upper panel) anti-CD3 stimulation. Each dot represents the mean value out of 3–5 independent experiments performed on a single T-cell clone. The mean ± SEM of each indicated sample group is shown. (B). Scatter plots of PD-1 expression *vs* GrB, TNF-α, IFN-γ expression, singularly or in co-production, showing a statistically significant inverse correlation with IFN-γ, GrB and polyfunctional capability. Each dot represents the mean percentage of each gp100 T-cell clone analyzed. The line shows the best fit to the data based on a simple regression model. Statistical analysis was performed with both Pearson (shown in Fig.) and Spearman tests (PD-1 *vs* GrB, R = 0.61, P = 0.03; PD-1 *vs* TNF-α, R = 0.37, = 0.22; PD-1 *vs* IFN-γ, R = 0.63, P = 0.02; PD-1 *vs* polyfunctionality, R = 0.58, $P = 0.04$). (C). Anti-PD-1 mAb blockade increases the polyfunctional capability. Bars represent the mean ± SEM of three independent experiments performed on Pt08.63 T cell clone (representative of three different clones tested).$^{*/\blacklozenge}$ $P \leq 0.05$, $^{**/\blacklozenge\blacklozenge}$ $P \leq 0.01$, $\blacklozenge\blacklozenge\blacklozenge$ $P \leq 0.001$, $^{****/\blacklozenge\blacklozenge\blacklozenge\blacklozenge}$ $P \leq 0.0001$, Mann-Whitney two-sample (*) test and two-tail Student's test ($\blacklozenge$), respectively.

resting-phase condition between the first and fourth round of stimulation, with overlapping results for each clone. To analyze whether the culture condition with IL-2 and stimulation may affect these molecule expression, we performed a time-course experiment in "resting" and activated conditions in two representative clones (Fig. S2). Although PD-1 and CD28 expression dramatically changed, their level was reverted after several days of stimulation by anti-CD3 mAb, to that observed without stimulus, indicating that the expression of the molecules that we reported in resting condition resembles their real expression (Fig. S2).

All Melan-specific $CD8^+$ T cells isolated before therapy (PRE) from the three patients displayed similar features in terms of phenotype and functionality (Fig. 4A, left panel). In particular, these cells expressed low levels of CD28 and PD-1 and were tumor-reactive and polyfunctional in terms of GrB and IFN-γ, the two effector molecules mainly involved in the anti-tumor-effector functionality of our clones (Fig. 4A). Only T cells isolated from Pt38 displayed a less differentiated phenotype as 25% of clones expressed high CD28 levels ($P = 0.03$, Kruskal-Wallis test). Notably, we found that the three patients showed a different baseline of Melan-A-specific TCR repertoire diversity, with Pt08 and Pt38 possessing a polyclonal, while Pt15 a restricted TCR repertoire (Fig. 4B).

When we analyzed the effect of the vaccine regimen on T-cell functionality, a different scenario was observed. In Pt08, treated with vaccination alone, the $CD8^+$ T cells specific for the two Ags displayed a different phenotype in terms of CD28 and PD-1 expression. In particular, Melan-A^+ T cells were negative for both CD28 and PD-1 immune-checkpoints, while $gp100^+$ T-cell clones were positive for both the molecules (Fig. 4A), suggesting a different grade of differentiation for these T cells. Notably, the increase of CD28 and PD-1 molecules was also evident in *ex-vivo* gp100-specific $CD8^+$ T cells of patient 08, collected before and after the vaccination treatment (Fig. S3). On the other hand, we found that Melan-A and gp100 peptides elicited a similar pattern of functionality and TCR diversity in this patient, as shown by the low anti-tumor activity and polyfunctionality, along with an oligoclonal TCR repertoire, for both gp100- and Melan-A-specific $CD8^+$ T cell clones (Fig. 4B and ref.[18]). Of note, in $gp100^+$ T cells the expression of PD-1 was directly involved in the dysfunctional anti-tumor activity, as indicated by the restoration of anti-tumor polyfunctionality by the anti-PD-1 blockade (Fig. 3C). Differently, Melan-A-specific low anti-tumor functionality was not attributable to PD-1 expression, these T cells being PD-1 negative.

In Pt15, treated with combined chemo-immunotherapy, who never recurred after the end of treatment (13 years), Melan-A elicited the expansion of late differentiated (CD28 negative), highly avid,[18] polyfunctional and tumor-reactive Ag-specific T cells, which showed an enhanced TCR diversity as compared to cells isolated before treatment. In these cells high levels of PD-1 did not impair anti-tumor functionality (Fig. 4A and ref.[16]). In this patient gp100 elicited the expansion of less differentiated $CD28^-PD\text{-}1^-$ Ag-specific T cells, with high anti-tumor activity, intermediate polyfunctionality and oligoclonal TCR repertoire (Fig. 4A, B).

In Pt38, treated with combined chemo-immunotherapy but who rapidly recurred, Melan-A induced the expansion of T cells with a heterogeneous phenotype and functionality, with the presence of both $CD28^+PD\text{-}1^-$ and $CD28^-PD\text{-}1^-$ T-cell subsets, along with high anti-tumor activity and polyfunctionality. However, in this patient we were unable to isolate the subset of $CD28^-PD1^+$ functional T cells identified in Pt15. The broad TCR repertoire displayed by Melan-A specific T cells before treatment was also found after therapy, although strong similarities were shared.[18] In this patient, gp100 elicited the expansion of heterogeneous but more differentiated $CD28^-PD\text{-}1^-$ and $CD28^-PD\text{-}1^+$ T cells, showing a high anti-tumor activity and polyfunctionality, along with a restricted TCR repertoire (Fig. 4B).

These data highlight how diverse Ags differently shape specific T-cell responses in the same patient.

Discussion

A major challenge in the designing of combined therapeutic strategies able to improve the efficacy of anti-tumor peptide-based vaccination is the identification of optimal $CD8^+$ T-cell populations characterized by Ag-specific TCR clonotype patterns and high anti-tumor functionality. The comprehension of

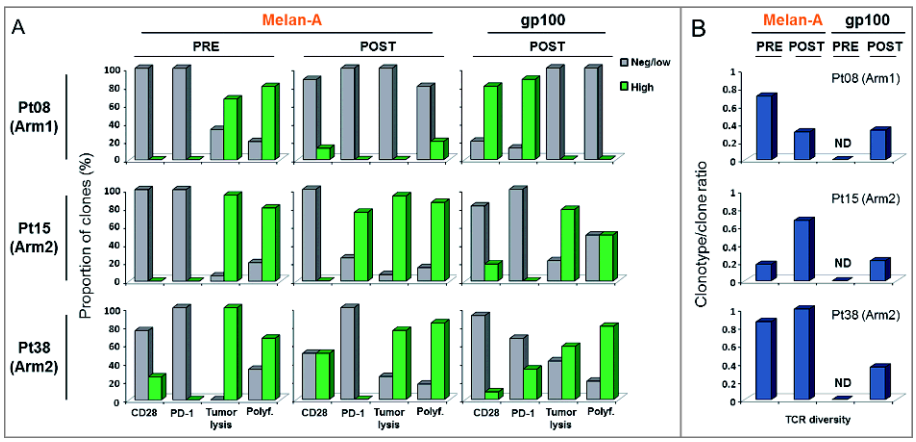


Figure 4. Comparison of phenotype, anti-tumor functionality and TCR repertoire in Melan-A- and gp100-specific $CD8^+$ T-cell clones isolated from the same patient. (A). CD28 and PD-1 expression, tumor lytic activity and polyfunctionality, in terms of GrB and IFN-γ, of all clones analyzed (n = 134), in Pt08, 15 and 38 grouped for Ag-specificity and time of PBMC collection (PRE and POST). A cut-off to discriminate between the negative/low and high value of each selected parameter was calculated, employing the mean value for all post-treatment clones, independently from Ag specificity, as follows: CD28, 14.7%; PD-1, 31%; tumor lysis, 37.9%; polyfunctionality, 53.5%. Histograms represent the relative percentage of clones that fall into the upper (green) or lower (grey) cut-off. (B). Histograms represent clonotype/clone ratio, as index of TCR diversity,[18] for Melan-A- (PRE and POST) and gp100-specific $CD8^+$ T-cell clones (POST).

how distinct tumor-associated Ags differently shape the TCR repertoire and T-cell functional activity in the same cancer patient may provide insight into the mechanisms underlying the biology of anti-tumor T-cell responses.

Herein, to identify whether the two different Ags (Melan-A/gp100) employed in a previously conducted peptide-based vaccination trial, alone or in combination with DTIC,[19] differently shape the anti-tumor response, we evaluated the anti-gp100 $CD8^+$ T-cell quality in the same patients previously analyzed for $CD8^+$ Melan-A specificity.[18] To this purpose we have performed a detailed phenotypical and functional analysis related to the TCR repertoire and clonality of treatment-driven anti-gp100 specific $CD8^+$ T cells and compared the anti-gp100 T-cell response to that previously reported for Melan-A, by analyzing the differences between the two Ags in influencing T-cell activity in the same patients.

The use of gp100 Ag in vaccination protocols for the treatment of melanoma has provided discordant results so far. Although different vaccination trials have reported a lack of a real advantage in the anti-tumor efficacy,[29,30] also in combination with the CTLA-4 blockade,[31] the $gp100_{209-}$ (210M)-based vaccination, in combination with IL-2, showed clinical benefits in metastatic melanoma patients.[32,33] At TCR level, Schrama et al. found the expansion of an oligoclonal gp100 210M-specific TCR repertoire from two patients receiving DTIC alone or in combination with low doses of IFN-α.[34] In agreement with these studies, in our study we observed that the 29 $gp100_{209-217}$ (210M)-specific clones sequenced from three patients were associated with a restricted TCR repertoire, irrespective of the treatment received (Fig. 1C). Of relevance, differently from available literature on $gp100^+$ T-cell clones, showing only TCRBV region families,[35-37] we extended our TCR analysis on CDR3 Vβ sequences, as a more accurate measure of TCR diversity. A detailed search in different public databases specific for TCR indicated that no $gp100_{209-217}$ TCR clonotype was recorded. Herein we identified nine new different clonotypes, not shared among the three patients evaluated, although we found high similarity in the CDR3 sequence of the ID9 clonotype of Pt38 with the only, to our knowledge, published $gp100_{209-217}$ specific sequence.[38] In all the patients evaluated we observed an expansion of patient-specific gp100 dominant clonotypes, irrespective of the treatment received (Fig. 1C and 2E). Although dominant clonotypes generally show high intrinsic Ag-avidity,[39] in our model no correlation was observed among clonal dominance and T-cell functionality. However, we evidenced that the combined chemo-immunotherapy expanded dominant clonotypes with high avidity and anti-tumor activity Fig. 2C-E), confirming that DNA-targeting drugs such as DTIC strongly influence the anti-tumor specific T-cell response.[16,18, 20,40-42]

While we cannot speculate as to whether the length of CDR3 may have any role in the functionality of our gp100-specific T cells, we observed that the clonotypes with the greatest CDR3 length showed the greatest frequency within the same patient Fig. 1C). The implications for TCRBV CDR3 length in Ag-specific immune response have not yet been completely elucidated, although it has been suggested to be dependent on thymic maturation, with positive selection being associated with shorter sequences and longer CDR3 with a higher affinity interaction with the peptide-MHC.[43]

At functional level, we found that DTIC plus peptide-vaccination elicited highly avid T-cell clones, able to recognize both modified and wild-type gp100 peptides, leading to an effective anti-tumor response and polyfunctionality, while vaccination alone induced low avid, poorly polyfunctional and non-tumor-reactive gp100-specific T cells (Fig. 2). Of note, these differences did not rely on TNF-α production, substantially low in all T-cell clones isolated from patients of both Arms (Fig. 2B), in agreement with the dispensable role of this cytokine in T-cell mediated anti-cancer activity.[44] It cannot be excluded that the treatment with DTIC might have improved the presentation of wild-type melanoma-associated peptides via up-regulation of HLA class I molecules, as recently demonstrated for temozolomide, which shares the same active compound with DTIC.[45]

Yet, a fraction of gp100- specific $CD8^+$ T-cell clones obtained after vaccination alone, although non tumor-reactive, showed a remarkable avidity either towards the wild type $gp100_{209-217}$ or the analogue 210M peptide (Fig. 2C, D), suggesting that the failure of the anti-tumor activity of these T cells might be ascribed to the expression of immune checkpoint inhibitory molecules.[46]

When we analyzed the expression of different inhibitory receptors, similar levels of LAG-3 and TIM-3 were observed irrespective of the treatment, although with a trend of an increasing expression of LAG-3 in T cells from the Arm1 patient, as we have already reported for Melan-A.[16] Differently, high PD-1 expression was observed almost exclusively in $CD8^+$ T cells isolated after vaccination alone (Fig. 3A). At functional level, PD-1/PD-L1 pathway engagement was directly involved with the low anti-tumor T-cell response of gp100-specific T-cells, as demonstrated by their ability to lyse the PD-L1 negative T2-peptide pulsed cells negative for PD-L1 expression (Fig. 2A, D) and the improvement of T-cell polyfunctionality following the PD-1 blockade (Fig. 3C).

A significant effort has recently been aimed at elucidating whether the differentiation status of T cells may be related to their phenotype and functionality. In particular, understanding how co-stimulatory and inhibitory signals are integrated in the generation of T-cell responses is critical for defining high quality T-cell responses. We found that a significantly high CD28 expression was detectable only in gp100-specific $CD8^+$ T cells isolated after vaccination alone (Table 1), along with high levels of the CXCR5 chemokine receptor (Table 1), high PD-1 levels (Fig. 3A) and poor anti-tumor functionality (Fig. 2A and B). This early differentiated effector memory $CD8^+CD28^{high}PD1^+CXCR5^+$ subgroup has recently been reported as the only proliferative subset after anti-PD-1 blockade in mice chronically infected with a Lymphocytic Choriomeningitis Virus.[26,27] Since these T cells are not functional in our model, further studies are needed to understand whether anti-PD1 treatment may be beneficial in patients possessing a substantial frequency of this T-cell subset. Differently, combined immunotherapy expanded anti-tumor functional $CD8^+$ T cell with low levels of CD28 and PD-1.

To evaluate how the nature of the Ag contributes to the shaping of the anti-tumor response in the same patient, herein we have compared phenotype, functionality and TCR

repertoire of all the gp100- and Melan-A-specific $CD8^+$ T cells collected in our study (Fig. 4). Our analysis revealed that chemo-immunotherapy was able to elicit highly functional Melan-A^+ and $gp100^+$ T cells possessing different phenotypes, $CD28^-PD\text{-}1^+$ and $CD28^-PD\text{-}1^-$ respectively. Differently, vaccination alone expanded poorly functional $CD28^-PD\text{-}1^-$ Melan-A^+ and $CD28^+PD\text{-}1^+$ $gp100^+CD8^+$ T cells. Remarkably our data indicate that only when PD-1 was expressed in the presence of CD28 it did confer an exhausted phenotype and defective anti-tumor functionality to the Ag-specific cells, reversible by PD-1 blockade (Fig. 3C), in accordance with recent studies showing that PD-1 regulates effector T-cell functionality primarily by inactivating CD28 signaling.[14] These observations indicate the relevance of the differentiation status and co-stimulatory/inhibitory immune-phenotype in the establishment of an effective Ag-specific anti-tumor response.

The molecular nature of the TCR and the clonotypic composition of the Ag-specific T-cell response emerge as critical determinants of anti-tumor $CD8^+$ T-cell function, as largely reported for viral models.[47] The role of TCR diversity, either in pre-existing or vaccine-driven repertoire, in the protective anti-tumor functionality, although evidenced in several models,[48] is still debated. Melan-A-vaccination, either with the wild type or analogue peptide, has been shown to favor the expansion of pre-existing dominant clonotypes in melanoma patients.[49,50] Moreover, treatment-driven TCR repertoire diversity has been related to different treatment vaccine formulations.[51,52] In melanoma, a highly restricted TCR repertoire has been linked to metastasis regression during cytokine therapy.[53] Neo-adjuvant Sipuleucel-T treatment of prostate cancer subjects has been shown to induce a narrowed TCR diversity in the periphery but enlarged in the tumor, with these differences reduced during the treatment, suggesting that vaccine-driven T cells were infiltrating the tumor.[54] In our settings, patients were clinically disease-free, thus we can suggest that the Melan-A broad repertoire found in the periphery,[18] rather than recruited in the tumor may persist in the circulation, exerting a patrol role against residual tumor cells, potentially preventing recurrence.

In our study, the comparison between gp100- and Melan-A-specific TCR repertoire diversity revealed different features, with an oligoclonal repertoire for gp100, irrespective of the treatment, differently from what we have previously reported for the broad Melan-A-specific response, observed only after chemo-immunotherapy.[18] We can speculate that the DNA damaging drug DTIC may have different effects on Ag-encoding genes, enabling the generation of novel Melan-A but not gp100 epitopes. Of relevance our analysis has been performed in the same patients, allowing the exclusion of the involvement of DNA repair enzyme O^6-methylguanine-DNA-methyltransferase (MGMT), responsible for the elimination of DTIC-mediated DNA adducts.[55] Of relevance, we found that the novel Melan-A clonotypes expanded by DTIC treatment are $PD\text{-}1^+CD28^-$ and highly functional. Consistently, Simon et al. have recently showed that PD-1 expression identifies Ag-specific T-cell clones with high avidity,[28] and Gros et al. described $PD\text{-}1^+$ neoantigen-specific anti-tumor $CD8^+$ T cells, either circulating or intratumoral.[56]

The different responses against the two Ags in the same patient can be also ascribed to: i) the peptide component of the MHC I/Ag complex, as described for epitopes derived from Epstein-Barr virus and Cytomegalovirus[57]; ii) the epitope dominance which results in the competition between Ag-bearing APCs and the Ag-specific $CD8^+$ T-cells.[58,59] In our model, both peptides were modified to increase the affinity for class I MHC-binding.[60,61] Therefore, since Melan-A and gp100 were injected at the same site, a local Ag-dependent cross competition of T cells with different specificities could have occurred.[62,63]

In conclusion, our data highlight how diverse Ags differently contribute to the shaping of peptide-vaccine-based specific T-cell response in the same patient, either in terms of immune phenotype or TCR repertoire. The implementation of cutting-edge technology at single T-cell level will clarify whether combined chemo-immunotherapy protocols including triazene compounds (i.e. DTIC and temozolomide), by impacting T-cell immunophenotype, TCR repertoire and anti-tumor functionality, will significantly induce a highly effective Ag-specific anti-tumor response.

Material and methods

Patients, cell lines and T-cell clones

Treatment of melanoma patients enrolled in Arm1 and Arm2 vaccination protocol has been previously described.[19] The pilot clinical study was approved by the Ethical Committees of the Regina Elena National Cancer Institute of Rome and the University of Rome "Tor Vergata". Pt.08 was vaccinated with Melan-A (A27L) and gp100 (210M) peptides plus IFN- (Arm1); Pt.15 and Pt.38 received the DTIC one day before the same vaccination schedule (Arm2). Peripheral blood mononuclear cells (PBMC) were isolated, upon informed consent, and cryopreserved as previously described.[19] T2 cell lines were purchased from the American Type Culture Collection. Melanoma cell lines Mel1 ($HLA\text{-}A2^-/gp100^+$), Mel2 ($HLA\text{-}A2^+$/gp100 and Mel3 ($HLA\text{-}A2^+/gp100^+$) were kindly provided by Dr. A. Anichini (Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy). Melanoma cell lines were isolated from surgical specimens of tumors of patients admitted to Fondazione IRCCS Istituto Nazionale dei Tumori, Milan. All lesions were histologically confirmed to be cutaneous malignant melanomas. Molecular and biological characterization of these cell lines has been reported previously.[64] Melanoma cell lines were periodically checked for the expression of a panel of tumor associated antigens (i.e. BRAF V600E, MITF, Tyrosinase, Melan-A, gp100, MAGE) by intracellular flow cytometry and western blot analysis. Cell lines were periodically checked (no more than 2 months from the assays) morphologically, and tested by growth curve analysis by ^{3}H-Thymidine incorporation assay and Mycoplasma detection. gp100-specific T-cell clones were generated by limiting dilution from pre-sensitized antigen-specific T-cell lines as described in ref.[18] and periodically re-stimulated (every 3 weeks) in RPMI 1640 medium supplemented with 10% human serum (noncommercial, prepared from a pool of healthy donors), 1 μg/ml PHA (Roche, 11082132001), 25 U/ml rIL-2 (Roche, 11147528001), and 1 $\times$ 10^6/ml

irradiated allogenic PBMC as feeder cells. T-cell clones were used 13-15 d after PHA-stimulation.

Antibodies and flow cytometry

Cell surface-staining was performed using various combinations of the following Abs from BD Pharmigen: PE-Cy7-CD27 (560809), PE-CD28 (555729), Alexa Fluor647-CXCR5 (558113), purified NKG2D (552886), PE-CD56 (340042), FITC-CD45RA (556626), PE-Cy7-CD45RA (560675), Alexa Fluor647-CCR7 (560816), PerCP-Cy5.5-PD-1 (561273), BB515-TIM-3 (565568), PE-LAG-3 (565616). For NKG2D staining a secondary PE-labeled anti-mouse antibody (Dako, R0439) was used. Intracellular staining was performed with the following mAbs from BD Pharmigen: PerCP-Cy5.5-TNF-α (560679), PE-Cy7-IFN-γ (557643), and Alexa Fluor647-GrB (560212). Ag-specificity of CD8+ T-cell clones was periodically tested by the use of FITC-CD8 (Miltenyi Biotec, 130-080-601) and PE-gp100 tetramers (Beckman Coulter, 082T01008/082T01012) as described.[19] The surface staining was performed for 30 min at 4°C. When possible, dead cells were excluded using propidium iodide staining (MP Biomedicals, 195458). When required, stimulation with plate-bound anti-CD3 mAb μg/ml) (CBT3 IgG2a),[65] was performed as indicated. For receptor blockade experiments, functional grade purified mAbs anti-PD1 (IgG1, 16-9989) and control Isotype (IgG1, 16-4714), both purchased from eBioscience, were used. The blockade was performed by incubation of T cells overnight with the corresponding blocking Ab (10 μg/ml) or with the control Isotype. Then cells were washed and co-cultured for the indicated time. For some experiments, the magnitude of staining was measured as integrated MFI (iMFI), a metric that combines the relative amount of molecule produced (the MFI) with the relative number of cells that make them (the percentage positive cells). iMFI is computed by multiplying the percentage with the MFI of positive cells and reflects the total functional response of a population.[16]

TNF-α, IFN-γ, GrB production and cytotoxicity assay

For intracellular staining (ICS) , gp100-specific T-cell clones were co-cultured with related (Mel3) or unrelated (Mel1 or Mel2) HLA/antigen melanoma cell lines, for 4-5 h at °C in the presence of the protein transport inhibitor GolgiStop (BD, 554724). T-cell clones were collected, washed with PBS, fixed using 2% paraformaldeide (15 min on ice) and then permealized by 5% FCS, 0,5% Saponine in PBS (20 min at RT). Cells were then incubated for 30 minutes at RT with PerCP-Cy5.5-TNF-α, PE-Cy7-IFN-γ and Alexa Fluor647-GrB mAbs. PD-1 blockade was performed as described above. Lytic activity and fine antigen recognition were assessed in a standard 4-hr Cr release assay as described.[18] Briefly, T2 cells, Mel1, Mel2 and Mel3 melanoma cell lines, pulsed or not with the analogue gp100 210M peptide (10 ug/ml) or the wild type $gp100_{209-217}$ peptide (10 ug/ml), with or without MHC-I blocking antibody W6-32,[66] were used as target cells. Cytotoxicity was performed by incubating ^{51}Cr-labeled target cells with effector cells at an E:T ratio of 20:1. Fine Ag specificity analysis was measured pulsing T2 target cells with decreasing concentrations of wild type $gp100_{209-217}$, analogue gp100 (210M) or irrelevant Melan-A peptides. The percentage of specific lysis was calculated as follows: 100 × (experimental release−spontaneous release)/(total release−spontaneous release).

TRBV repertoire analysis

The study of the TRBV repertoire was performed by amplifying the RNA extracted from gp100-specific T-cell clones with a TCR beta (TRB) variable consensus primer , followed by sequencing of the amplified products, as described in ref. [18] One μg of total RNA, extracted from gp100-specific $CD8^+$ T-cell clones, was used to prepare the specific TRB chain first strand cDNA with AMV Reverse Transcriptase (AMV RT; Promega Corp) and TRBC-1 primer, specific for a TRB constant region recognizing both TRBC1 and TRBC2 genes. cDNA was subjected to PCR amplification using TRBC-2 primer and TRBVc variable consensus primer, designed to amplify TRB chain rearrangements containing virtually all the known human TRBV genes. PCRs were performed under the following conditions: 93°C for 1 min, 52°C for 1 min, 72°C for 1 min with the last extension cycle performed at 72°C for 7 min. Sequencing reactions were run on the ABI 3700 Prism automated capillary sequencer (Applied Biosysystem) starting from eight ng of purified PCR products using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and a TRBC primer (TRBC-3) recognizing a sequence internal to that amplified with the consensus PCR. The sequence of all primers and probes used in this work are listed in ref..[18] TCR sequences were compared with published TRBV, TRBD, TRBJ and TRBC segments[67] and the CDR3 was established according to the IMGT unique numbering (CDR3-IMGT) proposed by Lefranc et al.[68]

Statistical analysis

Continuous variables were expressed as mean ± standard deviation. Values are reported as mean ± SEM/SD or median (range). Student's t-test, Mann-Whitney U test or Kruskal-Wallis test (when appropriate) were used to compare quantitative variables, as previously reported.[16,69] The Bonferroni correction was used to adjust for multiple-comparison. Pearson chi-square test was used to compare proportions. The correlation analysis was performed with Spearman and Pearson test. P value $\leq$ 0.05 was considered significant. Statistical analyses were carried out using SPSS software (SPSS version 21, SPSS Inc., Chicago, IL, USA).

Abbreviations

Ag	antigen
Ags	antigens
APCs	antigen presenting cells
CDR3	complementarity-determining regions 3
CTL	cytotoxic T lymphocyte
CXCR5	C-X-C chemokine receptor type 5
DTIC	dacarbazine
EC50	half-maximal lysis
GrB	granzyme-B

ICOS	inducible co-stimulator
ICS	intracellular staining
IFN	interferon
LAG-3	lymphocyte-activation gene 3
NKG2D	Natural Killer Group 2D
PBMC	peripheral blood mononuclear cells
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
PHA	phytohemaglutinin
Pt	patient
TCR	T cell receptor
TIM-3	T-cell immunoglobulin and Mucin Domain-3
TNF	tumor necrosis factor
TRBV	T Cell Receptor Beta Variable

Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the authors.

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