ORIGINAL ARTICLE

PBMC are as good a source of tumor-reactive T lymphocytes as TIL after selection by Melan-A/A2 multimer immunomagnetic sorting

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Abstract Choosing a reliable source of tumor-specific T lymphocytes and an efficient method to isolate these cells still remains a critical issue in adoptive cellular therapy (ACT). In this study, we assessed the capacity of MHC/peptide based immunomagnetic sorting followed by polyclonal T cell expansion to derive pure polyclonal and tumor-reactive Melan-A specific T cell populations from melanoma patient's PBMC and TIL. We first demonstrated that this approach was extremely efficient and reproducible. We then used this procedure to compare PBMC and TIL-derived cells from three melanoma patients in terms of avidity for Melan-A A27L analog, Melan-A$_{26-35}$ and Melan-A$_{27-35}$, tumor reactivity (lysis and cytokine production) and repertoire. Regardless of their origin, i.e., fresh PBMC, peptide stimulated PBMC or TIL, all sorted populations (from the three patients) were cytotoxic against HLA-A2+ melanoma cell lines expressing Melan-A. Although some variability in peptide avidity, lytic activity and cytokine production was observed between populations of different origins in a given patient, it differed from one patient to another and thus no correlation could be drawn between T cell source and reactivity. Analysis of Vβ usage within the sorted populations showed the recurrence of Vβ3 and Vβ14 subfamilies in the three patients but differences in the rest of the Melan-A repertoire. In addition, in two patients, we observed major repertoire differences between populations sorted from the three sources. We especially documented that in vitro peptide stimulation of PBMC, used to facilitate the sort by enriching in specific T lymphocytes, could significantly alter their repertoire and reactivity towards tumor cells. We conclude that PBMC which are easily obtained from all melanoma patients, can be as good a source as TIL to derive high amounts of tumor-reactive Melan-A specific T cells, with this selection/amplification procedure. However, the conditions of peptide stimulation should be improved to prevent a possible loss of reactive clonotypes.

Keywords Melan-A · MHC/peptide · Immunomagnetic sorting · Melanoma · Adoptive immunotherapy

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Introduction

Adoptive cell therapy (ACT) of cancer patients consists in the isolation, in vitro expansion and reinjection of tumor-reactive lymphocytes. While initial ACT trials in melanoma were performed with tumor-infiltrating lymphocytes (TIL) of undefined specificities [14, 24] the extensive characterization of numerous melanoma antigens has allowed the development of new ACT protocols targeting defined epitopes. Among melanoma-associated antigens recognized by tumor specific CTL, Melan-A is the most

commonly expressed antigen by melanoma tumors and Melan-A epitopes presented in the HLA-A0201 context are the most frequently recognized by tumor infiltrating lymphocytes from melanoma patients [2, 17]. Therefore most of the recent ACT protocols have targeted these epitopes and documented encouraging immunological and clinical responses [8, 27, 28]. A remaining challenge to further improve these protocols is to design and validate a fast and simple method to obtain pure reactive Melan-A cytotoxic T cells from PBMC or TIL from all melanoma patients.

In some trials, Melan-A specific effector cells were peripheral blood T lymphocyte populations enriched by in vitro peptide stimulation [18, 20]. However, since none of these populations were pure, this strategy has the major drawback of injecting activated uncharacterized T cells together with the specific effectors. Purity was achieved in other studies by injecting T cell clones derived from these populations [27, 28] but this strategy presents the risk of picking a clone that, despite its selection in vitro, may turn out to be poorly active in vivo against the tumor. Furthermore, the cloning step and the subsequent expansion of a clone is a lengthy process requiring multiple in vitro stimulations that may drive the clone towards senescence and impair its reactivity in vivo [9, 10, 13, 21]. Because of these concerns, we chose immunomagnetic sorting with HLA/peptide coated beads [3] to isolate pure but yet polyclonal Melan-A specific T cells. The first issue of our study was to evaluate the efficiency of this method to achieve the expansion of polyclonal and tumor reactive T cells in a reproducible manner.

The second issue is the choice of a reliable source of tumor specific T lymphocytes and in this respect, PBMC and TIL (whether from tumor-invaded lymph nodes or from metastatic lesions) from melanoma patients have been used to obtain tumor specific T cells [8, 18, 20, 27, 28]. Although TIL from patients are frequently enriched in melanoma specific T cells [2, 17] and have been used with some success in ACT protocols [7, 16], they are very difficult to isolate except from invaded lymph nodes. The most convenient source of these cells would therefore be PBMC that can be obtained from all patients. Yet, differences in Melan-A/A2 repertoires from PBMC or TIL from the same patient have been reported [5, 19, 25] that could result in significantly different functional properties of Melan-A specific T cells from the two sources. In addition, in vitro stimulation of PBMC with peptide which has been used in these studies to increase the fraction of Melan-A reactive lymphocytes may further alter repertoire diversity. To formally evaluate whether Melan-A specific lymphocytes sorted from PBMC can be as good effectors as those sorted from TIL, we compared the function and repertoire of Melan-A specific T cell lines selected by MHC-multimer sorting from tumor-invaded lymph nodes (TILN) and from PBMC previously enriched or not in specific T cells by peptide stimulation.

Materials and methods

Cell lines

Melanoma cell lines "M" were established in our laboratory from metastatic tumor fragments as previously described [11]. The human mutant cell line CEMx721 T2 (T2) used as presenting cell was a generous gift from T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium).

Production of melanoma TIL

Short-term cultured TIL (gift from the unit of cellular and gene therapy of Pr. B. Dreno, Nantes) were isolated by culturing cryopreserved fragments of stage III metastatic lymph nodes into 12-well tissue culture plates with X-Vivo 15 serum-free medium (Bio*Whittaker, Walkersville, MD, USA) containing 150 U/ml rIL2 (Eurocetus, Rueil-Malmaison, France) and glutamine (1 nM) (Bio*Whittaker) for 10–14 days and then expanded on feeder cells as previously described [14].

Construction of HLA-A*0201/peptide tetramers

HLA-A0201/peptide α3-mutated monomers were generated as previously described [3]. Recombinant proteins were produced as inclusion bodies in E. coli XA90F'Lac^{Q1}, dissolved in 8 M urea, and refolded with 50 μg/ml of Melan-A A27L peptide (ELAGIGILTV), purchased from Eurogentech (Brussels, Belgium). Tetramerisation was performed as previously described [3]. Briefly, HLA monomers were biotinylated for 4 h at 30°C with 6 μg/ml BirA (Immunotech), purified on a monoQ column (Pharmacia, St Quentin en Yvelines, France) and tetramerised with PE-labeled streptavidin (Sigma, St Louis, MO, USA) at a molar ratio 4/0.8.

Tetramer staining

To minimize non-specific staining, HLA-A2-Melan-A tetramer was tittered and used at the lowest concentration that allowed the detection of 100% of a Melan-A specific T cell clone diluted among PBMC. PBMC or TIL were incubated for 1 h at 4°C in the dark with Melan-A tetramer (10 μg/ml). After washing in PBS-0.1% BSA, cells were resuspended in PBS and analyzed on a FACScan.

Immunomagnetic cell sorting and expansion of T cell sorted populations

HLA-A*0201/Melan-A monomers (20 µg/ml) were incubated for 1 h at room temperature with 6.7×10^6 streptavidin-coated beads (Dynabeads M-280 streptavidin, DYNAL, Compiegne, France) and washed in PBS/0.1% BSA. 5×10^6 PBMC were rotated for 4 h at 4°C with monomer-coated beads [3]. After ten washes, bead coated cells were expanded using a polyclonal T cell stimulation protocol [14].

Positive magnetic cell sorting of Vβ8 and Vβ14 specific cells was performed with Dynabeads (Dynal Biotec, Compiegne, France). Briefly, 5×10^6 lymphocytes were incubated with 15 µg/ml mouse anti-human Vβ8 or Vβ14 antibodies (Immunotech, Beckman-Coulter, Marseille, France) on ice for 45 min, washed twice with PBS/0.1% BSA and resuspended in culture medium. Subsequently cells were incubated with sheep anti-mouse IgG coated Dynabeads (Dynal Biotec, Compiegne, France) at a 1:1 ratio for 4 h at 4°C with gentle rotation. Rosetted Vβ + T cells were trapped by a magnet (MPC 6, Dynal Biotec) and washed ten times in PBS/0.1% BSA. The cell/bead suspension was incubated in culture medium in 6-well plates overnight at 37°C to allow beads to detach. After overnight incubation, beads were extracted by the magnet, and sorted lymphocytes were transferred on feeder cells, as previously described [14].

Briefly, 2000 bead-coated T cells/well were distributed in 96-well plates mixed with irradiated feeder cells [LAZ EBV-B cells (2×10^4/well) and allogeneic PBMC (10^5/well)], in 150 µl of culture medium supplemented with IL-2 (150 U/ml) and PHA (15 µg/ml).

Synthetic peptides

The Melan-A peptides $_{27–35}$ AAGIGILTV and $_{26–35}$ EAAGIGILTV and the decamer analogue A27L ELAGIGILTV were purchased from Eurogentech (Brussels, Belgium). Purity (>70% for biologic assays and >80% for HLA-A2 folding) was controlled by reversed-phase high-performance liquid chromatography. Peptides were lyophilized, dissolved in DMSO at 10 mg/ml and stored at –80°C.

PBMC stimulation

PBMC from HLA-A2 stage III melanoma patients were stimulated with irradiated allogeneic HLA-A2 melanoma line pulsed with the Melan-A A27L analogue, in presence of 5 ng/ml IL-6 (Sigma, St louis, USA) and 5 ng/ml IL-12 (Sigma), according to a method previously described [12].

Irradiated and peptide-loaded stimulator cells were added again twice, at day 7 and 14, in presence of 10 U/ml IL-2 and 5 ng/ml IL-7.

^{51}Chromium cytotoxicity assay

Cytotoxic activity was measured in a standard 4-h assay against ^{51}Cr-labeled cells. Briefly, target cells (peptide pulsed-T2 or melanoma cells) were incubated with 100 µCi $Na_2^{51}CrO_4$ (Oris Industrie, Gif-sur-Yvette, France) at 37°C for 1 h. Effector cells were then added at an E/T ratio of 10/1, in a final volume of 100 µl. For peptide recognition assays, T2 cells were preincubated with a range of Melan-A peptide concentrations for 1 h at 37°C. After the 4 h-co-culture, 25 µl of supernatant were mixed with 100 µl of scintillation cocktail (Optiphase Supermix, Wallak, UK) for measurement of radioactive content.

Cytokine production assay

About 1×10^5 lymphocytes were stimulated by 3×10^5 stimulator cells (melanoma cells and peptide-pulsed T2 cells) in 200 µl of RPMI 1640-10% FCS in the presence of brefeldin A, 10 µg/ml (Sigma, St Louis MO, USA) in round-bottom 96-well plates. The cultures were incubated for 6 h at 37°C in 5% CO_2 humidified atmosphere. For intracytoplasmic cytokine staining, cells were then fixed 10 min at room temperature in a solution of PBS 4% paraformaldehyde (Sigma), washed and stored at 4°C until labeling. Fixed stimulated lymphocytes were stained for cytokine production using the method described by Jung et al. [15]. Anti-IL2, anti-TNF and anti-IFN-γ specific antibodies were purchased from BD Biosciences, France. After staining, cells were resuspended in PBS and 1.5×10^4 events were analyzed on a FACScan flow cytometer using Cell Quest software (Beckton Dickinson, Grenoble, France).

TCR β-chain V region expression analysis

A panel of 24 anti-Vβ Abs was used in this study (Immunotech, Beckman-Coulter, Marseille, France). Anti-Vβ1, –2, –3, –4, –5.1, –5.2, –5.3, –6, –7, –8, –9, –11, –12, –13.1, –13.2,–13.6, –14, –16, –17, –18, –20, –21.3, –22 and –23 mAbs were used as purified mAbs. Staining and washing were performed in PBS, 0.1% BSA. For indirect fluorescence labeling, cells were incubated (1) with purified anti-Vβ mAbs for 30 min at 4°C and washed, (2) with goat anti-mouse FITC-labeled Ab for 30 min at 4°C and washed. Cells were then analyzed by flow cytometry.

Results

Evaluation of MHC/peptide multimer sorting and expansion to amplify Melan-A specific polyclonal populations from TIL or PBMC

We tested the efficiency and reproductibility of MHC/ multimer immunomagnetic sorting on either TIL or PBMC from HLA-A2 melanoma patients. Experiments were performed in duplicate for each tested sample. The fraction of tetramer labeled cells in the starting TIL population was high (9%) but hardly detectable in the two PBMC samples. We obtained almost pure tetramer-labeled populations from each of these samples following one or two sort/amplification cycles (Fig. 1a). The enrichment factor in specific T cells was very similar in duplicated experiments, thus

documenting the reproducibility of this sort/amplification method. Moreover, the enrichment factor could be as high as 1940 when starting from PBMC which underlines the efficiency of this strategy (Table 1). In order to evaluate a possible effect of sort/amplification cycles on repertoire diversity, the pattern of TCR Vβ usage of sorted populations was determined. First of all, as shown on Fig. 1b, the Melan-A specific repertoire was very similar in duplicate experiments in all three patients strongly documenting the reproducibility of the procedure. Furthermore, the high degree of polyclonality of the Melan-A specific population obtained from patient 2 after two rounds of sort/amplification suggested that this procedure did not majorly alter repertoire diversity. Finally, we checked the tumor reactivity of each sorted population on HLA-A2 melanoma cell lines expressing the target antigen. All the sorted

Fig. 1 **a** Sorting of Melan-A specific lymphocytes from TIL and PBMC. HLA-A*0201/ Melan-A-A27L monomers coated on magnetic beads were used to isolate in duplicate Melan-A-specific populations from melanoma patients' PBMC or TIL. PE-conjugated tetramers were used to assess the purity of the sorted populations after a 14-day amplification on feeder cells. *Values* indicate the percentage of cells labeled with tetramers after one or two rounds of sorting/amplification. **b** Repertoire diversity of sorted populations was assessed by labeling with 24 anti-Vβ antibodies. Inserts indicate the percentage of the specific population characterized with this panel in each experiment: *white bars* experiment 1, *black bars* experiment 2. **c** Lysis of melanoma cell lines by Melan-A sorted populations: white and black symbols correspond to duplicate experiments. *Circles* and *squares* represent two HLA-A2 melanoma cell lines expressing the Melan-A antigen and lozenge a HLA-A2 negative melanoma cell line
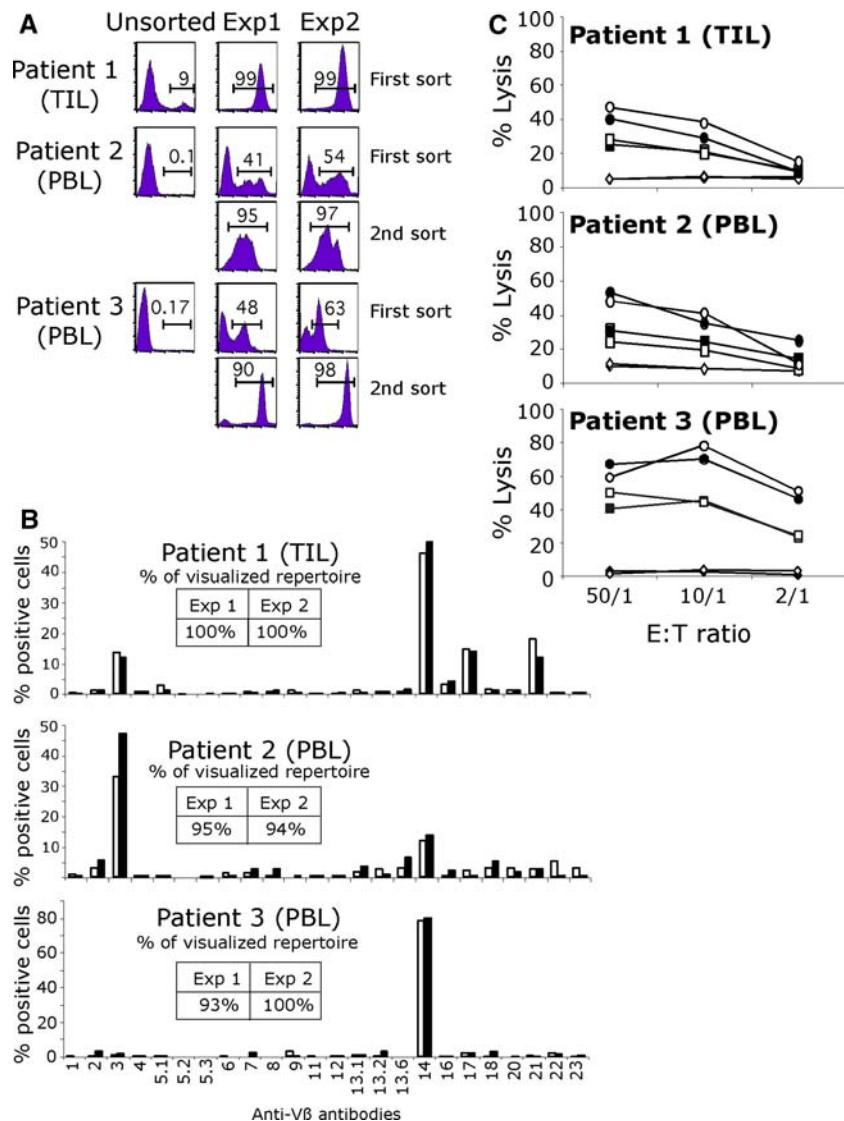
Table 1 Enrichment and amplification yields in Melan-A specific T cells after MHC/peptide multimer sorting

	Patient 1 (TIL)		Patient 2 (PBL)		Patient 3 (PBL)	
Starting population						
Total cell number	4×10^6		5×10^6		5×10^6	
CD8 specific cells (%)[a]	9		0.1		0.17	
CD8 specific cells nb[b]	3.6×10^5		5×10^4		8.5×10^4	
First sort	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
D14 after sort/amplification						
Amplified cell number	1.2×10^8	1.3×10^8	2.2×10^7	1.8×10^7	2×10^7	2×10^7
CD8 specific cells (%)[a]	99	99	41	54	48	63
CD8 specific cell nb[b]	1.19×10^8	1.29×10^8	9×10^6	9.7×10^6	9.6×10^6	1.2×10^7
Enrichment[c]	×330	×358	×1,800	×1,940	×1,129	×1,482
Second sort						
Total cell number			5×10^6	5×10^6	5×10^6	5×10^6
Nb of specific cells[b]			2×10^6	2.7×10^6	2.4×10^6	3.1×10^6
D14 after sort/amplification						
Amplified cell number			3.2×10^8	2×10^8	2.5×10^8	2.5×10^8
CD8 specific cells (%)[a]			95	97	90	95
CD8 specific cells nb[b]			3.1×10^8	1.6×10^8	2.3×10^8	2.4×10^8
Enrichment[c]			×155	×61	×96	×76

[a] % of Melan-A specific T cells is determined by CD8/tetramer double labeling

[b] Number of specific cells (Percentage of tetramer labeled cells multiplied by the total number of cells)

[c] Enrichment factor is the ratio between the number of specific cells after and before each sort/amplification cycle

populations were cytotoxic against these cell lines, and in accordance with the results obtained on repertoire diversity, tumor reactivity was very similar in duplicate experiments (Fig. 1C). Finally, these highly enriched populations remained extremely stable upon multiple restimulations in terms of purity and tumor reactivity (data not shown).

Together these results validated the choice of such a procedure to obtain Melan-A specific populations from blood or TIL. In particular, the demonstration of reproducibility was a prerequisite to be able to compare PBMC and TIL as alternate sources of Melan-A specific T cells, for use in immunotherapy.

Comparison of TIL and PBMC from three melanoma patients as a source of Melan-A specific T cells

We used PBMC, Melan-A$_{A27L}$-stimulated PBMC and TIL from 3 HLA-A2 melanoma patients, as different sources of Melan-A specific lymphocytes. PBMC stimulation was performed by three rounds of co-incubation with an irradiated HLA-A2 positive Melan-A negative melanoma cell line loaded with the Melan-A$_{A27L}$ analog [12].

Before sorting, tetramer positive cells were hardly detectable in unstimulated PBMC, but easily detected among TIL (0.09–15%) and peptide-stimulated PBMC

(8.2–45%). In 4/9 populations derived from PBMC or TIL, a single sort with monomer-coated beads was sufficient to obtain highly enriched (over 90%) tetramer-labeled cells (Fig. 2). Following polyclonal amplification, a second sort was performed on the remaining five partially enriched populations and resulted in a purity above 90% in every case (Fig. 2). Thus we were able to obtain almost pure tetramer-positive cell-lines from all patients with at most two multimer sorts, regardless of the starting populations (unstimulated or stimulated PBMC or TIL).

Each sorted T cell line was then tested for peptide specificity and tumor reactivity. To this end, the relative avidity of sorted populations was first evaluated by determining their EC50 against peptide-loaded T2 cells, using a standard ^{51}Cr release assay. In all patients, sorted populations displayed higher avidities towards both decapeptides, i.e. the analog decapeptide Melan-A A27L (EC50 ranging from 0.05 to 10 nM) and the natural Melan-A$_{26–35}$ decapeptide (EC50 from 0.04 to 20 nM), as compared to their avidity towards the natural Melan-A$_{27–35}$ nonapeptide (EC50 from 1 to 500 nM) (Fig. 3). Although this may have resulted in part from the fact that the peptide used to sort and/or stimulate Melan-A specific lymphocytes was a decamer, we have previously shown that this decamer was the preferential epitope recognized by clones derived from TIL [23].
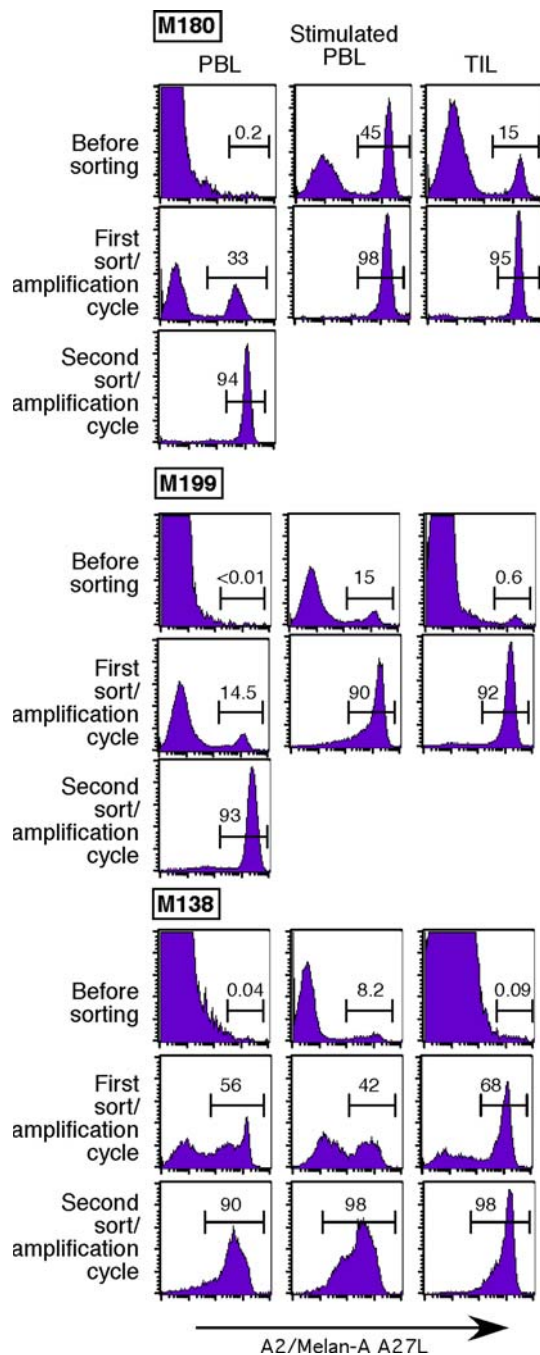
Fig. 2 Sorting of Melan-A specific lymphocytes from different sources. HLA-A*0201/Melan-A-A27L monomers coated on magnetic beads were used to isolate Melan-A-specific populations from unstimulated PBMC, peptide stimulated PBMC and TIL in three melanoma patients. PE-conjugated tetramers were used to assess the purity of the sorted populations. *Values* indicate the percentage of cells labeled with tetramers after one or two rounds of sorting/amplification steps

avidity of the three sorted populations towards the different Melan-A peptides were rather similar. In patient M138 (middle panel), lymphocytes sorted from TIL exhibited a higher avidity towards the three Melan-A peptides, whereas in patient M199 (lower panel), antigen-specific lymphocytes sorted from unstimulated PBMC displayed a higher avidity than those sorted from TIL or stimulated PBMC. In conclusion, we did not evidence any preferential source to isolate Melan-A specific lymphocytes of high avidity.

Since our final goal was to generate tumor-reactive T cells, the populations under study were also compared for their cytotoxicity and cytokine production in response to HLA-A2 melanoma cell lines that spontaneously express the Melan-A antigen.

All sorted T cell lines were lytic against A2$^+$/Melan-A$^+$ melanoma cell lines and produced IFN-γ and TNF upon stimulation by these cells (Fig. 4). For each patient, the tumor reactivity of the T cell lines was correlated with their relative avidities towards Melan-A peptides. For example, in patient M138 (hatched bars), tetramer-specific lymphocytes sorted from TIL were the most reactive against melanoma cells whereas, in patient M199 (black bars), cells sorted from unstimulated PBMC were the most reactive (especially in terms of cytokine production). Therefore, tumor-reactivity of sorted populations could not be predicted from their origin. Nonetheless, it is interesting to note that a significant fraction of IL-2 producing cells, a characteristic of highly reactive anti-tumor lymphocytes [12, 27], was observed in all three populations sorted from stimulated PBMC, whereas IL-2 producing cells were present in only one out of three and two out of three populations sorted from unstimulated PBMC and TIL, respectively.

The pattern of TCR Vβ usage of sorted populations was then assessed to determine if repertoire differences could explain their different avidities and tumor reactivities. To this aim, we used a panel of 24 anti-Vβ antibodies representing the most frequently expressed Vβ chains within a normal repertoire. Using this panel of antibodies, the mean fraction of accessible repertoire in our patients was $83 \pm 12\%$ (inserts in Fig. 5). Sorted populations were rather polyclonal and a similar degree of diversity in Vβ usage was observed in unstimulated or stimulated PBMC or TIL in two patients (M180 and M199). In contrast, patient M138 displayed a more restricted repertoire, particularly among peptide-stimulated PBMC (hatched bars). However, in this last sample, Melan-A/A2 Vβ TCR diversity was probably underestimated since only 63% of the MelanA repertoire was detected by our panel of antibodies.

A number of recurring Vβ subfamilies were observed among the three patients: Vβ2, Vβ3, Vβ14, Vβ16 and

We did not observe any clear correlation between the avidity of sorted populations and their origin (TILN or PBMC). Indeed, in patient M180 (upper panel), the relative

Vβ17 (Fig. 5). Concerning patient M180, the results were in accordance with the similar avidity and reactivity of the three sorted populations, i.e., there were no marked difference between Vβ subfamilies of the sorted populations according to their origins (Fig. 5, upper panel). Sorted populations from the two other patients were more heterogeneous in term of Vβ subfamilies. Concerning patient M138 (middle panel), Vβ7 subfamily was dominant among unstimulated PBMC (white bars), but very low or absent among TIL and stimulated PBMC, respectively. Concerning patient M199, Vβ8 T cells were dominant in Melan-A specific T cells sorted from unstimulated PBMC (white bars), but absent among those derived from TIL and from stimulated PBMC. In contrast, the Vβ14 subfamily was dominant among cell populations derived from peptide-stimulated PBMC (hatched bars), but hardy detectable in nonstimulated PBMC.

In order to check if the different avidities diplayed by PBMC and stimulated PBMC from this last patient (Figs. 3, 4) could be due to the variable proportion of distinct Vβ subfamilies (Fig. 5), the dominant subpopulations Vβ8 and Vβ14 were sorted and then analyzed for their avidity for peptide-loaded T2 cells and their reactivity towards melanoma cells.

Relative avidity of Vβ-sorted populations from M199 patient

Figure 6a illustrates the purity of these sorted populations in terms of Vβ expression and tetramer labeling. As shown on Fig. 6b, Vβ8 lymphocytes derived from unstimulated PBMC were of higher avidity towards Melan-A peptides than Vβ14 cells derived from peptide-stimulated PBMC. Indeed, EC50 of the Vß8 population were respectively, of 0.1, 30 and 100 nM on Melan-A A27L, Melan-A$_{26-35}$ and $_{27-35}$ whereas EC50 of the Vβ14 sorted population were of 30 nM, 1 μM and 500 nM on these same peptides. Finally, and in accordance with results obtained on Melan-A peptides, the Vβ8 sorted population was more reactive on Melan-A/A2 melanoma lines than the Vβ14 population, as illustrated by cytokine production in response to melanoma cells (Fig. 6c).

Discussion

The aim of this study was to compare unstimulated PBMC, peptide-stimulated PBMC and TIL from lymph nodes as alternate sources of functional Melan-A specific lymphocytes. To this aim, we first validated a procedure based on

Fig. 3 Avidities of Melan-A-specific sorted CTL lines for Melan-A peptides. The TAP-deficient cell line T2 expressing HLA-A*0201 was loaded with various concentrations of either the natural peptides Melan-A$_{26-35}$ (*filled square*) or Melan-A $_{27-35}$, (*open square*) or the modified decapeptide A27L (*filled circle*). Lytic activity was evaluated at an E:T ratio of 10:1 by the classical 4-h ^{51}Cr release assay
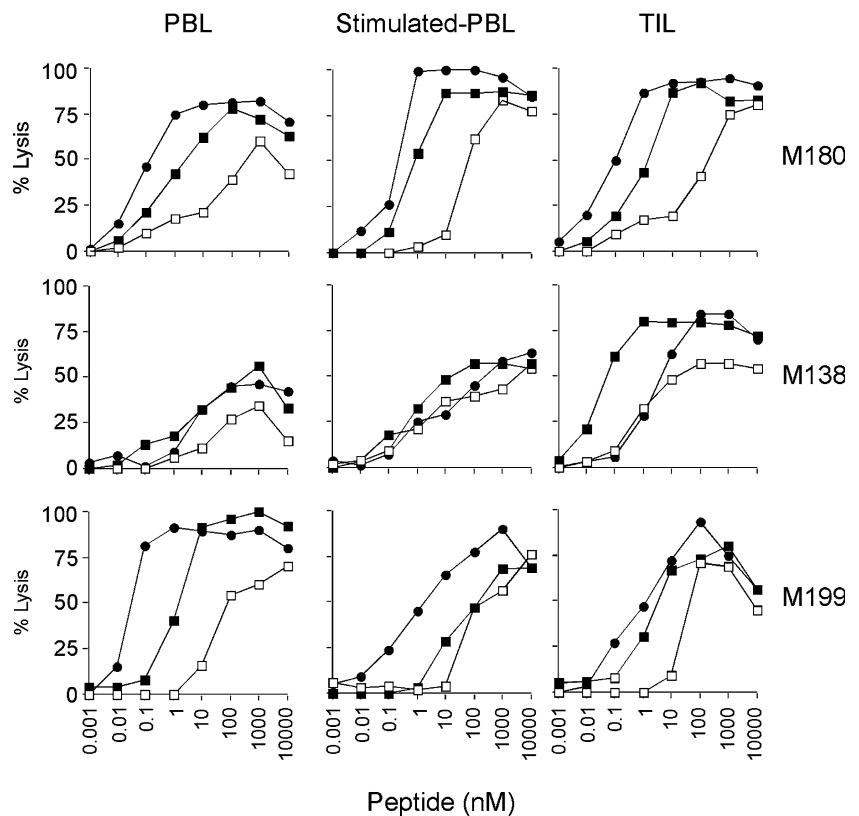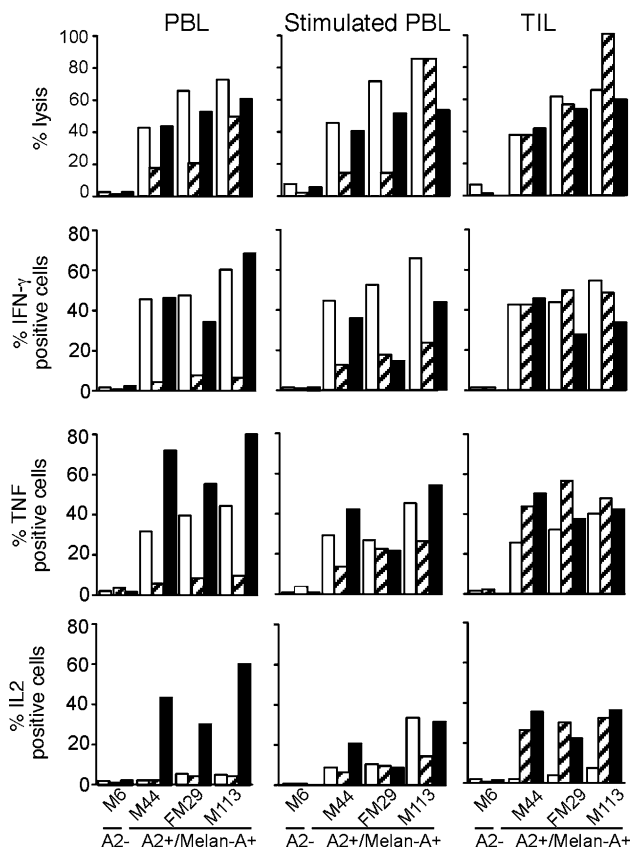
Fig. 4 Reactivity of Melan-A specific sorted populations towards melanoma cell lines: *open square* patient M180, (*hatched bars*) patient M138, *filled square* patient M199. For lysis experiment, effector and target cells were incubated at a 10:1 ratio. For cytokine production, effector and target cells were incubated at a 1:2 ratio in the presence of Brefeldin A, stained with anti-cytokine antibody and analyzed by flow cytometry (5×10^3 cells)

immunomagnetic sorting of specific T cells with MHC/peptide coated beads. The peptide used to generate MHC/peptide complexes and to stimulate PBMC in vitro was the well-characterized Melan-A A27L analog peptide, previously shown to cross-stimulate a large fraction of the T cell repertoire directed against the natural Melan-A epitopes [26].

We demonstrated that this method was efficient to isolate and amplify Melan-A-specific populations hardly detectable by standard tetramer staining. These populations were polyclonal, as assessed by Vβ staining, and tumor-reactive (Fig. 1). Moreover, we documented the high reproducibility of this method by comparing duplicate experiments. Thus, this sorting procedure can be used to analyse the repertoire and function of the whole Melan-A specific population in contrast to the previously reported analyses of clones [5, 19] which can be biased by the variable ability of these clones to proliferate in vitro.

Using this procedure, we were able to obtain over 90% pure Melan-A specific populations from PBMC with two rounds of sorting/expansion whereas a single round was most of the time sufficient to achieve the same purity when starting with peptide-stimulated PBMC or TIL (Figs. 1a, 2). A second round of sorting/expansion did not seem to alter the diversity of the Melan-A repertoire since, as could be documented in patients M180 and M199, a similar number of Vβ subfamilies were obtained from PBMC (two sorts) or from stimulated PBMC and TIL (one sort). This Vβ diversity of the Melan-A repertoire, the general dissimilarity of the repertoire between patients and the recurrence of Vβ3 and Vβ14 subfamilies are in accordance with previous reports [5, 6, 19, 25].

In each of the three patients, a global analysis revealed a similar pattern of Vβ usage in cells sorted from PBMC, stimulated PBMC or TIL although in variable proportions. Nonetheless, we sometimes observed the major expansion of a particular Vβ family in only one of the three sources from the same patient. For example, in patients M138 and M199, Vβ7 and Vβ8 subfamilies were predominant in PBMC while Vβ13.2 and Vβ14 subfamilies were over-represented in stimulated PBMC, respectively. Likewise, Vβ3 subfamily was preferentially expressed in cells sorted from TIL in patient M138 (Fig. 5). Considering that those expanded subfamilies represented a high proportion of MelanA/A2 sorted cells, we wondered whether their preferential expansion in a single sample could result in functional differences between sources in the same patient. As a matter of fact, we did observe differences in reactivity of specific T cells from each source when challenged with Melan-A peptides and melanoma cell-lines, especially in terms of cytokine production. For example, in patient M138, initial peptide stimulation of PBMC dramatically increased the recovery of cytokine producing specific T cells while TIL remained the most reactive towards peptide and melanoma cells (Fig. 4, hatched bars). In contrast, in patient M199, T cells derived from unstimulated PBMC were more reactive than those derived from peptide-stimulated PBMC or TIL (Figs. 3, 4, black bars). In this last patient, peptide stimulation resulted in the complete disappearance of the dominant Vβ8 subfamily and dramatically favored the expansion of the Vβ14 subfamily (Fig. 5). After sorting of these Vβ8 and Vβ14 subfamilies, we observed a significant difference between these two populations in terms of avidity and melanoma reactivity (Fig. 6b, c). This result underlines the fact that in vitro peptide stimulation can majorly affect the Melan-A repertoire leading to either a decrease (patient M199) or an increase (patient M138) in global reactivity towards melanoma cells, especially when the initial

Fig. 5 Analysis of the Vβ repertoire of sorted Melan-A specific cells from unstimulated PBMC (*open square*), stimulated PBMC (*hatched bars*) and TIL (*filled square*) patients. A panel of 24 anti-Vβ Abs was used. *Insets* indicate the percentage of the specific population characterized with this panel in each patient
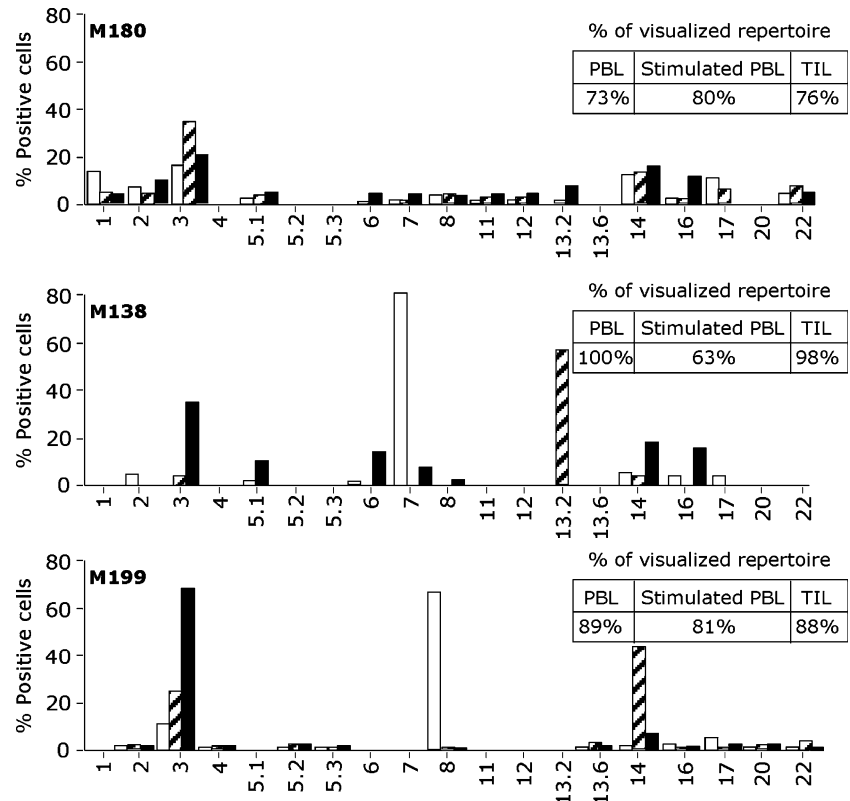
repertoire in PBMC is very oligoclonal, as seen in those two patients. In contrast, in patient M180 whose initial Melan-A repertoire in PBMC was the most diverse, peptide stimulation had little effect on repertoire and reactivity. We assume that adjusting the concentration of stimulating peptide and the number of stimulations should allow the recovery of a broader reactive repertoire. Indeed, it has been reported that the amount of peptide used to pulse antigen-presenting cells for in vitro stimulation influences the avidity of peptide specific CTLs [1, 29].

An alternative explanation for the loss of highly reactive T cells would be that multimer sorting itself induced apoptosis of these cells due to a prolonged stimulation of their TCR, as previously reported following incubation of mouse T cells with soluble MHC tetramers at 37°C [4]. In our procedure, the sort is performed at 4°C, thus limiting T cell activation, and then, sorted cells are immediately restimulated with PHA, IL2 and feeder cells. Thus, although apoptosis of some cells certainly occurred, it is impossible for us to delineate precisely which stimuli triggered apoptosis and especially the contribution of persistent multimer stimulation in this process. Nonetheless, we documented that Melan-A specific T cells with high tumor reactivity can be efficiently sorted and expanded from all samples using this procedure, despite a

potential loss of some reactive T cell clones. In support of that, a recent study compared the sorting of Melan-A specific T cells using either reversible multimers or classical tetramers and concluded that "reversible multimer-based isolation was not better than the tetramer-based T cell isolation method regarding the generation of CTL clones with superior peptide avidity and/or tumor recognition efficiency" [22].

Finally, multiple cycles of stimulations have been shown to drive specific T lymphocytes towards replicative senescence [9, 10, 13, 21] which may alter their in vivo efficiency after transfer. Thus, as future developments, we consider using a single round of PBMC stimulation with low concentrations of peptide followed by MHC-peptide multimer sorting to obtain enriched Melan-A specific populations at an early stage of differentiation.

In conclusion, we demonstrated that PBMC from melanoma patients can provide a reliable source of Melan-A reactive T lymphocytes that were as good effectors as those derived from TILN and that HLA/peptide multimer sorting is a very efficient method to isolate pure population from this source. We reckon that this strategy could be extended to other melanoma epitopes in other HLA contexts and could thus represent a promising approach for the development of future ACT protocols.
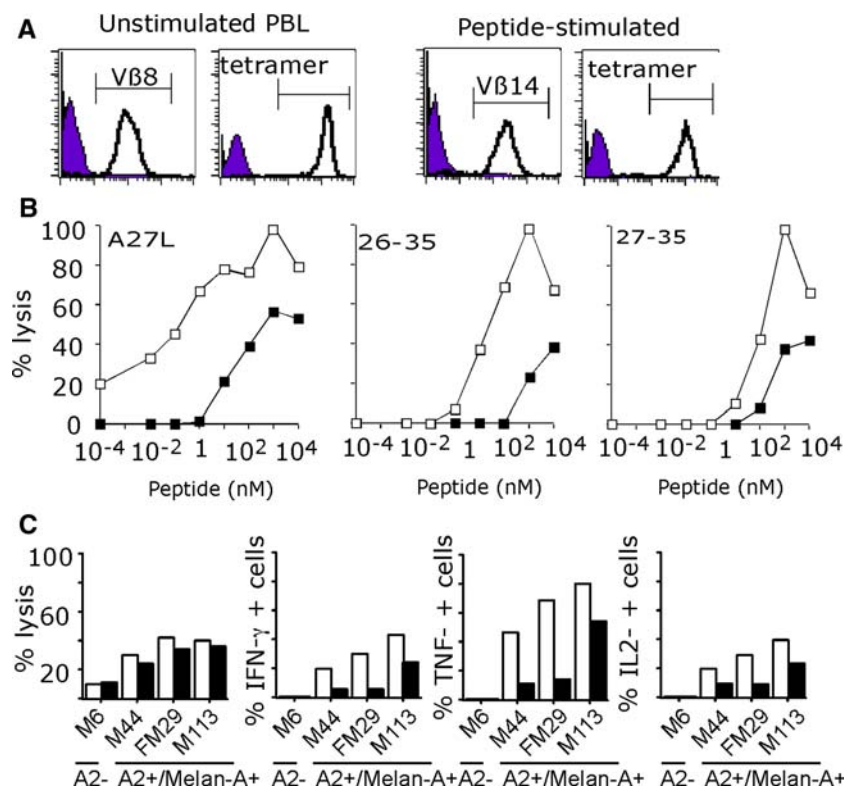
Fig. 6 Analysis of MelanA specific Vβ8 and Vβ14 T lymphocytes from patient M199. **a** Vβ and tetramer staining of Vβ sorted populations. **b** Avidities of Vβ8 (*open square*) and Vß14 (*filled square*) sorted T cells for MelanA peptides. The TAP-deficient cell line T2 was loaded with various concentrations of either the modified decapeptide A27L or the natural peptides Melan-A$_{26–35}$ and Melan-A $_{27–35}$. Lytic activity was measured at an E:T ratio of 10:1 by 4-h ^{51}Cr release assay.

c Lysis and cytokine production by Vβ8 (*open square*) and Vβ14 (*filled square*) sorted populations in response to HLA-A2 Melan-A positive or negative melanoma cells. For lysis experiment, effector and target cells were incubated at a 10:1 ratio. For cytokine production, effector and target cells were incubated at a 1:2 ratio in the presence of Brefeldin A, stained with anti-cytokine antibody and analysed by flow cytometry (5×10^3 cells)

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