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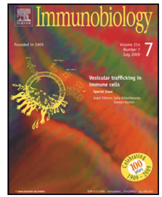

Melanocortin 1 Receptor-derived peptides are efficiently recognized by cytotoxic T lymphocytes from melanoma patients

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ABSTRACT

Background: Melanocortin 1 Receptor (MC1R) is expressed in a majority of melanoma biopsies and cell lines. We previously demonstrated that three hydrophobic low-affinity HLA-A2-restricted MC1R-derived peptides: $MC1R_{291-298}$, $MC1R_{244-252}$ and $MC1R_{283-291}$ can elicit cytotoxic T-lymphocytes (CTL) responses from normal donor peripheral blood lymphocytes (PBL). Moreover, peptide-specific CTL recognized a panel of MHC-matched melanomas, demonstrating that human melanoma cell lines naturally present MC1R epitopes. However, the natural presence of MC1R-specific T cells in melanoma patient's tumour and blood remains unknown.
Methods: The presence of anti-MC1R specific $CD8^+$ T cells was established in a population of melanoma-specific T cells derived from peripheral blood mononuclear cells (PBMC) and tumour-infiltrating lymphocytes (TIL) from HLA-$A2^+$ melanoma patients.
Results: CTLs specific for the three MC1R-derived peptides that lysed allogeneic HLA-$A2^+$$MC1R^+$ melanomas were elicited from PBMC, demonstrating the existence of an anti-MC1R T cell repertoire in melanoma patients. Moreover, TILs also recognized MC1R epitopes and HLA-$A2^+$ melanoma cell lines. Finally, HLA-A2/$MC1R_{244}$-specific $CD8^+$ T cell clones derived from TILs and a subset of $MC1R_{291}$ specific TILs were identified using HLA-A2/MC1R tetramers.
Conclusion: Our results demonstrate that MC1R-derived peptides are common immunogenic epitopes for melanoma-specific CTLs and TILs, and may thus be useful for the development of anti-melanoma immunotherapy.

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Introduction

Cancer immunotherapy is mainly based on the capacity of $CD8^+$ cytotoxic T lymphocytes (CTLs) to recognize major histocompatibility complex class I (MHC-I)-restricted tumour-associated antigens (TAAs) on malignant cells (Brichard et al., 1993; Kawakami et al., 1994a,b; Restifo et al., 2012; Smyth et al., 2006; Van Der Bruggen et al., 2002). The identification of a large number of MHC-I-restricted melanoma-associated antigens (MAA) has considerably increased the capacity to develop cell/peptide-based immunotherapy against malignant melanoma (MM) (Coulie et al., 1994; Palucka and Banchereau, 2012; Rosenberg et al., 1998). Since most MAAs are derived from proteins closely related to tissues of melanocytic origin that are normally expressed in melanosomes and probably involved in regulation of melanogenesis (Adema et al., 1994; Chen et al., 1995; Kawakami et al., 1994c; Sakai et al., 1997; Winder et al., 1994), greater attention has been directed towards other melanoma/melanocyte-related proteins to identify novel MAA candidates. One such protein is the human melanocyte hormone stimulating Melanocortin 1 Receptor (MC1R) (Chhajlani and Wikberg, 1992). The 35 kDa MC1R protein, which belongs to a subgroup of the G-protein-coupled receptor family, is mainly located in melanoma and melanocytes (Tatro et al., 1990; Xia et al., 1996). Expression of MC1R in several other human tissues including adrenal and pituitary glands, cerebellum, placenta, testis and *in vitro* activated monocytes/macrophages and dendritic cells

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(DC) has also previously been demonstrated, although to significantly lower levels compared to melanoma cells (Lopez et al., 2007; Salazar-Onfray et al., 2002; Thornwall et al., 1997).

A major hurdle that remains to be addressed in peptide-based immunotherapy is the induction of T cell tolerance, since the majority of human TAAs are derived from endogenous (self)-proteins (Lopez et al., 2009). It should also be noted that the most-well studied HLA-restricted TAAs could be considered as relatively dominant, with a higher MHC stabilization capacity compared to the large majority of TAAs presented on tumour cells (Brichard et al., 1993; Chen et al., 1995; Kawakami et al., 1994a,b,c). Moreover, several human studies using DC-based vaccination with dominant MHC-restricted TAAs resulted in moderate effects on melanoma patient survival rate compared to treatments with adoptive cell transfer or DC-based vaccinations using tumour lysates (Aguilera et al., 2011; Knutson et al., 2002; Lopez et al., 2009; Parkhurst et al., 2004; Schaed et al., 2002; Slingluff et al., 2001; Vonderheide et al., 2004). Other studies based on mouse models demonstrated that the observed lack of efficacy was mainly due to induction of tolerance in the available T cell repertoire towards MHC-I in complex with dominant TAAs (Colella et al., 2000; Gross et al., 2004; Grossmann et al., 2001; Hernandez et al., 2000). Furthermore, the vast majority of the characterized TAAs is not broadly expressed nor even involved in tumour cell proliferation and survival. Consequently, therapeutic strategies that target TAAs that are not involved in tumour cell growth could result in the selection of aggressive cancer clones that do not express these specific antigens (Schmollinger et al., 2003; Wenandy et al., 2008).

In contrast, T cell self-tolerance concerns mainly MHC-restricted dominant self-epitopes but rarely low-affinity epitopes (Parkhurst et al., 1996; Valmori et al., 1998). Furthermore, this latter ensemble of T cells is composed of frequent CTLs with high avidity towards MHC-I-restricted low-affinity epitopes, efficiently inducing *in vivo* anti-tumour immunity (Gross et al., 2004). Thus, since the CTL repertoire specific for these epitopes might have escaped the negative selection process, it should be possible to recruit high-avidity CTLs against MHC-I-restricted low-affinity epitopes resulting in potent antitumour responses. We have previously identified the three highly hydrophobic nonameric MC1R-derived peptides $MC1R_{291}$ (AIIDPLIYA), $MC1R_{244}$ (TILLGIFFL) and $MC1R_{283}$ (FLALIICNA) that bind with low affinity to HLA-A2 and can activate peptide-specific CTLs in PBMC from healthy HLA-A2^{+} donors (Salazar-Onfray et al., 1997). Moreover, the elicited MC1R-specific CTLs recognized efficiently HLA-A2^{+}MC1R^{+} melanoma cells, demonstrating that these MC1R-derived epitopes are naturally processed and presented by HLA-A2 on the surface of melanoma cells (Salazar-Onfray et al., 1997).

In this study, the presence of anti-MC1R-specific precursors was evaluated in a population of CTLs derived from peripheral blood mononuclear cells (PBMC) and anti-MC1R CD8^{+} T cells in tumour-infiltrating lymphocytes (TIL) populations, both obtained from melanoma patients. While five CTL lines recognized HLA-A2 in complex with either $MC1R_{244}$ or $MC1R_{291}$, two CTL lines also recognized HLA-A2/$MC1R_{283}$. Surprisingly, a predominant amount of all assessed TILs displayed significant cytotoxic activity against the three MC1R epitopes in complex with HLA-A2. Furthermore, three CD8^{+} T cell clones, isolated from one TIL (TIL-1206), killed efficiently target cells loaded with the $MC1R_{244}$ peptide. Importantly, $MC1R_{291}$-specific CD8^{+} T cell subsets were also identified using HLA-A2/$MC1R_{291}$ tetramers in TILs isolated from melanoma biopsies. Taken together, our results demonstrate that several low-affinity HLA-A2-restricted MC1R-derived epitopes can initiate significant *in vitro* and *in vivo* T cell responses against melanoma cells. Consequently, we believe that these epitopes could be useful in future immunotherapy approaches against melanoma.

Materials and methods

Patients

Tumour tissue and/or blood samples were obtained from patients with advanced (stage IV) malignant melanoma (MM) treated at Radiumhemmet, Karolinska University Hospital. All the protocols used in this study were approved by the Bioethical Committee of the Karolinska University Hospital, Karolinska Institute, Stockholm, Sweden and by the Bioethical Committee for Human Research of the Faculty of Medicine, University of Chile, Santiago, Chile.

Cell lines

Besides the HLA-A2^{+} melanoma cell lines FM3D and FM55.M1, both kindly provided by Dr. J. Zeuthen (Cancer Society, Copenhagen, Denmark), all the melanoma cell lines (DFB, DFW, FMS, AK, BL, DL, 0549, AA, and AMK) used within this study were established at the Department of Microbiology, Tumour and Cell biology (MTC) (Karolinska Institute, Stockholm, Sweden) and maintained at the Institute of Biomedical Sciences (ICBM), University of Chile (Supplementary Table 1). The TAP-deficient T2, the class I-defective and HLA-A2-transfected C1R-A2, the colon cancer SW480 and the ovarian cancer OVA3507 cell lines were purchased from ATCC (Manassas, VA, USA).

Synthesis of HLA-A2-restricted melanoma associated peptides

All the melanoma-associated and control peptides (Supplementary Table 2) were synthesized according to standard solid-phase methods and purified using high performance liquid chromatography (HPLC) as previously described (Kawakami et al., 1994a). The purity and identity of peptides were determined by analytical HPLC and mass-assisted laser desorption ionization mass spectrometry. The peptides were dissolved at a concentration of 5 nM in DMSO (Sigma–Aldrich) and stored at $-20\,^{\circ}C$ until further use. The stabilization capacity of the different peptides was evaluated as previously described (Allerbring et al., 2012).

Generation of peptide-specific T cells

PBMC from HLA-A2^{+} melanoma patients were isolated by Ficoll/Hypaque density-gradient centrifugation (Pharmacia UpJohn, Uppsala, Sweden). A total of 5×10^{7} cells from the isolated PBMC were seeded in 75 cm^{2} flasks in a serum free medium (AIM-V, Life technologies, Paisley, UK) in a total volume of 10 mL and incubated for 2 h at $37\,^{\circ}C$. Non-adherent cells (PBL) were gently removed and cryopreserved with FBS 10% DMSO, and thereafter stored in vapour-phase liquid nitrogen until time for CTL generation. Adherent monocytes were maintained in culture during 7–10 days in AIM-V medium containing 500 IU/mL recombinant human interleukin 4 (rhIL-4) (US-Biological, Swampscott, MA, USA), and 800 IU/mL recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) (Shering Plough, Brinny Co., Ireland). Cells were fed every second day with fresh medium supplemented with rhGM-CSF and rhIL-4.

The antigen presenting cells (APCs) were matured 48 h before harvesting with 4.25 ng/mL recombinant human tumour necrosis factor alpha (rhTNF-α), 4.25 ng/mL recombinant human interleukin-6 (rhIL-6), 17 ng/mL recombinant human interleukin-1 beta (rh-IL-1β) and 8.5 ng/mL prostaglandin E_2 (PGE_2). APCs were harvested, counted and 3×10^{5} cells/mL were loaded with 1 μg/mL different melanoma associated antigens-derived peptides (Supplementary Table 2), $MC1R_{291}$, $MC1R_{244}$, $MC1R_{283}$, and $gp100_{280-288}$, or the control peptide MP_{58-66}, and incubated at $37\,^{\circ}C$ for 4 h. Then,

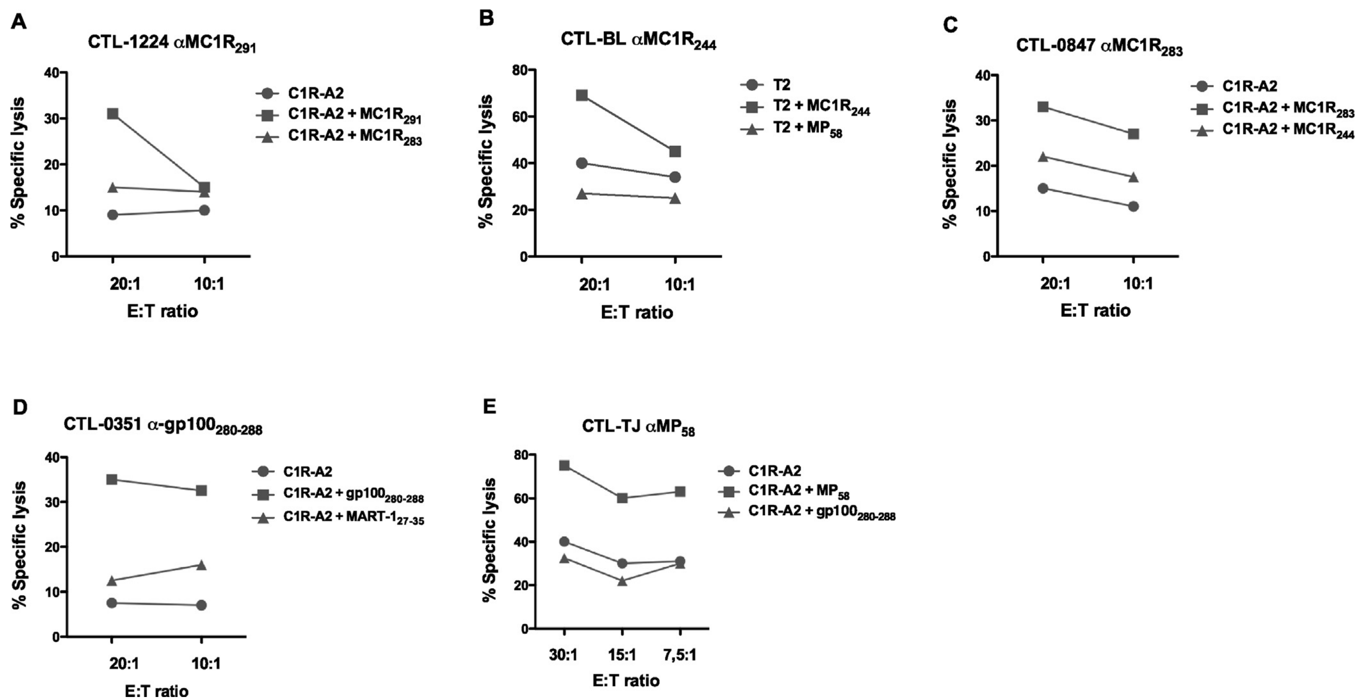

Fig. 1. The three highly hydrophobic low-affinity HLA-A2-restricted MC1R peptides induce efficient CTL responses from melanoma patient PBMC with similar amplitude to more dominant epitopes. The cytotoxicity of several CTL lines derived from PBMC of melanoma patients was tested against peptide-pulsed HLA-A2+ T2 or C1R-A2 target cells using a standard ^{51}Cr release assay. Similar results were obtained at least two times with independently produced CTLs for each peptide. (A–C) The HLA-A2/MC1R$_{291}$, HLA-A2/MC1R$_{244}$ and HLA-A2/MC1R$_{283}$-specific CTL lines 1224, 0847 and BL, respectively, recognized efficiently target cells C1R-A2 or T2 loaded with cognate MC1R peptides. Conversely, target cells alone or loaded with control peptides were not recognized. (D–E) The HLA-A2/gp100$_{280–288}$ and HLA-A2/MP$_{58}$-specific CTL lines 0351 and TJ recognize efficiently C1R-A2 target cells coated with gp100$_{280–288}$ and MP$_{58}$, respectively. Conversely, target cells alone or loaded with control peptides were not recognized.

frozen autologous PBL from patients were thawed and co-cultured for 7–10 days with the peptide-loaded APCs (T cells:APC ratio 20:1) in RPMI 1640 medium (Invitrogen) containing 10% FBS (Invitrogen) and nourished every second day with 5 ng/mL rhIL-6 (eBioscience) and 0.4 ng/mL rhIL-12 (ProSpec-Tany TechnoGene). T cells were re-stimulated with peptide-loaded autologous APC at a 30:1 ratio, and maintained thereafter with 100 IU/mL rhIL-2 and 10 ng/mL rhIL-7 for an additional period of 10 days. The following 2nd and 3rd stimulations were performed every 7–10 days using previously cryopreserved peptide-loaded autologous monocytes or T2 cells when autologous cells were not available. After four stimulations, the CTLs were tested for specific recognition of target cells using a standard ^{51}Chromium release assay.

Tumour-infiltrating lymphocytes (TIL), melanoma-specific CTLs and melanoma cell lines derived from melanoma metastasis

Single cell suspensions obtained from fresh HLA-A2+ metastatic tumours and tumour-infiltrated lymph nodes were cultured at a concentration of 1.5×10^6 cells/mL in RPMI supplemented with 10% FBS and 50 IU/mL rhIL-2 (for TILs cultures) or 1 μg/mL cyclosporine A (for melanoma cell lines cultures). Cells were stimulated every third day with the same supplemented media. In some cases, the TILs were stimulated once with irradiated autologous melanoma cells during the second week of *in vitro* culturing. The adherent fraction of cells was isolated early and cultured in RPMI supplemented with 10% FBS. The cells were maintained in culture until confluence. Melanoma cell lines were obtained after approximately ten culture passages. PBL-derived CTL lines specific for HLA-A2+ melanoma cells were obtained after four rounds of weekly stimulation with autologous tumour cells. Briefly, 10×10^6 PBMCs from one MM patient (code BL) were periodically incubated with irradiated autologous melanoma cells at an effector:target (E:T) ratio of 20:1 in the presence of 50 IU/mL of rhIL-2. The IL-2 was always added 12 h after tumour stimulation. For tetramer staining, TILs were previously expanded trough a rapid expansion protocol. Briefly, 2.5×10^6 TILs were cultured in T75 tissue culture flask (BD Biosciences, California, USA) in a total volume of 45 mL of CellGro (CellGenix, Freiburg, Germany) medium supplemented with 2% human serum (Lonza, Basel, Switzerland) and 30 ng/mL of OKT3 (AbCam Cambridge, UK), and with 40 Gy irradiated feeders pooled from at least two donors in a 1:100 ratio. 24 h after, 5 mL of 3000 U/mL IL-2 (Peprotech, USA) in a final concentration of 300 U/mL were added. At day 4, 50% of the medium was exchanged to fresh medium containing 600 U/mL IL-2 (Peprotech, USA). When the density was deemed high enough (at least 1×10^6/mL, approximately 7–10 days), the cells were separated into two T75 flasks with twice the volume of medium supplemented with 600 U/mL IL-2. At days 10–13, cells were transferred to culture bags with 50% of new medium containing 600 U/mL IL-2. At day 14, cells were harvested and stained with HLA-A2 tetramers. The cytotoxic activity of CTLs derived from PBMC and TILs was measured against T2 or C1R-A2 cells exogenously loaded with peptides (1 μg/mL) as well as against autologous melanoma cells. K562 and HLA-A2− melanoma cell lines were used as negative controls in ^{51}Cr-release assays that were performed after 2–3 weeks *in vitro* culture. All the TIL clones were obtained from a line established from one single patient (TIL-1206) using standard limiting dilution.

Cytotoxic assays

Melanoma, C1R-A2 or T2 cells were used as targets. C1R-A2 and T2 cells were loaded with 2 μg/mL of each peptide, incubated for 2 h at 26 °C and used thereafter in a standard ^{51}Cr-release assay. Cytotoxicity assays were performed by incubating ^{51}Cr-labelled target cells (1×10^6 melanoma cells, peptide-pulsed T2 cells or C1R-A2 cells) with effectors cells at various effector:target ratios for 4 h at 37 °C. Supernatants were harvested and radioactivity was

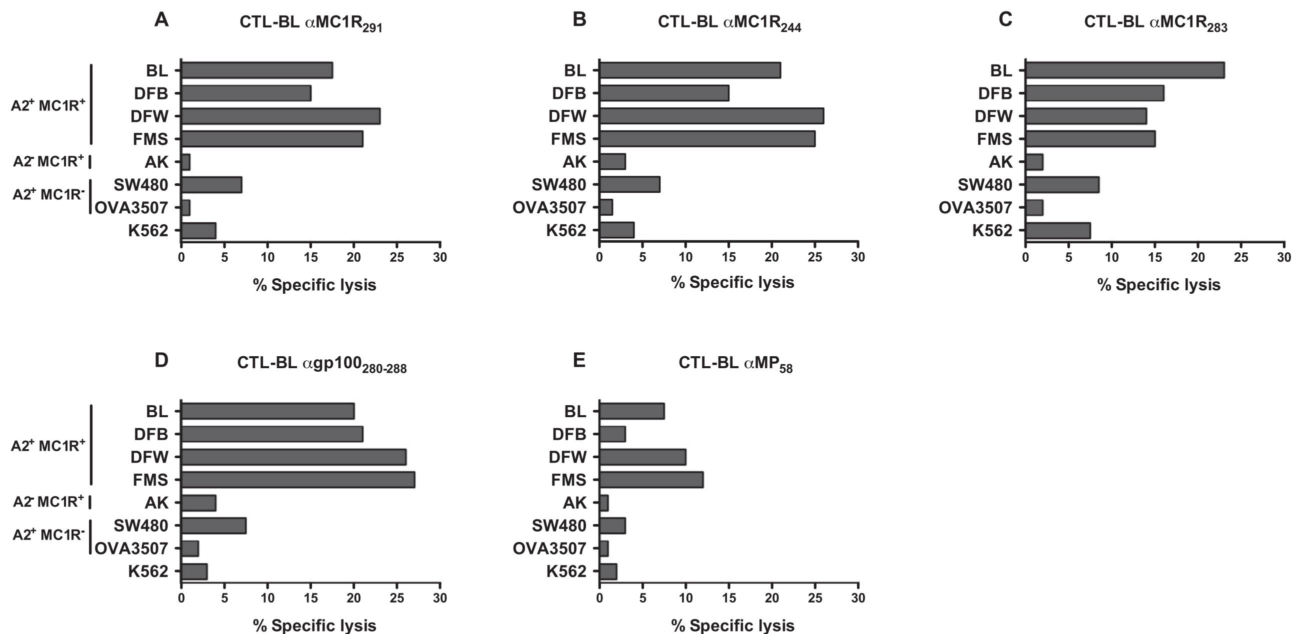

Fig. 2. HLA-A2/MC1R-specific CTL lines derived from a melanoma patient recognize efficiently HLA-A2$^+$MC1R$^+$ melanoma cells. CTL lines elicited against each of the MC1R peptides (Panels A–C) or against the dominant melanoma-associated gp100$_{280-288}$ or the viral MP$_{58-66}$ peptides (Panels D and E) were tested in an standard ^{51}Cr release assay at an effector/target ratio of 20:1 against the autologous HLA-A2$^+$MC1R$^+$ melanoma cell line BL, the three allogeneic HLA-A2$^+$MC1R$^+$ melanoma cell lines DFB, DFW and FMS as well as the HLA-A2$^-$MC1R$^+$ melanoma cell line AK. The control HLA-A2$^+$MC1R$^-$ ovarian carcinoma and colon carcinoma cell lines OVA3507 and SW480 were not recognized. All assays were performed with an excess of K562 cells, demonstrating a marginal effect of NK cell activity.

determined using a gamma counter (Wallac, Uppsala, Sweden). The percentage of ^{51}Cr release was calculated according to the following formula: % lysis = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). To eliminate lymphokine-activated killer (LAK) cell activity or unspecific natural killer (NK) cell-mediated cytotoxicity of the obtained cell lines, all the cytotoxicity assays were performed in the presence of an excess of cold K562 cells to every ^{51}Cr-labelled target cell in a saturation ratio of 20:1.

Preparation of HLA-A2/MC1R tetramers

Refolding and tetramerisation of MC1R-derived and controls (HIV$_{77-85}$ and MART-1$_{27-35}$) pMHC complexes were performed as previously described (Michaelsson et al., 2000). Due to its very high hydrophobicity, we were unable to produce sufficient amounts of pure MC1R$_{283}$ peptide required for MHC refolding and production of tetramer. HLA-A2 molecules containing BirA substrate peptide (bsp) were refolded with human β$_2$m and the peptides MC1R$_{244}$ or MC1R$_{291}$. Both MHC complexes were thereafter purified and enzymatically biotinylated with the BirA enzyme and biotin (Avidity, Denver, CO, USA). Biotinylated pMHCs were then mixed with streptavidin (BD Biosciences) and conjugated with Phycoerythrin (PE) at a 4:1 molar ratio.

Analysis of anti-MC1R T-cell populations

TILs obtained from patients' biopsies were stained with HLA-A2/MC1R$_{244}$, HLA-A2/MC1R$_{291}$ and control HLA-A2 tetramers presenting either the HIV$_{77-85}$ or MART-1$_{27-35}$ epitopes. Cells, gated using anti-CD3 and anti-CD8 antibodies (eBioscience, San Diego, CA), were analyzed using a FACScalibur cytometer (Becton Dickinson, Franklin Lakes, NJ). Tetramer positive cell populations were gated considering HIV as a negative control for each sample. Despite the fact HIV-specific cells can be detected using tetramers in peripheral blood of HIV seronegative individuals (Alanio et al., 2010; Legoux et al., 2010), we consider that the percentage of HIV positive cells in TILs populations isolated from melanoma metastases could be considered zero. The data were evaluated using the FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Results

Efficient recognition of melanoma cell lines by in vitro elicited HLA-A2/MC1R-specific CTLs

Using PBMC from healthy donors, we have previously demonstrated that the hydrophobic low affinity nonameric HLA-A2-restricted melanoma-associated MC1R-derived epitopes MC1R$_{244}$, MC1R$_{283}$ and MC1R$_{291}$ induce specific CTL recognition of T2 cells or HLA-A2-transfected C1R cells pulsed with each MC1R peptide (Salazar-Onfray et al., 1997). In order to assess whether PBMC from malignant melanoma (MM) patients also contained CTL precursors directed against these three MC1R epitopes, CTL lines from PBMC of five HLA-A2$^+$ melanoma patients were induced using autologous peptide-loaded APCs. At least two specific CTL lines were obtained for each of the analyzed MC1R-derived peptide epitopes. All the three MC1R epitopes induced efficient CTL activity against HLA-A2-matched target cells pulsed with the same MC1R peptide (Fig. 1A–C). Interestingly, the amplitude of the CTL responses was comparable to those elicited by more dominant HLA-A2-restricted peptides such as the MAA gp100$_{280-288}$ or the influenza virus-derived MP$_{58-66}$ (Fig. 1D and E). Importantly, none of the elicited MC1R-specific CTL lines recognized unpulsed target cells, nor target cells pulsed with irrelevant HLA-A2-restricted peptides (Fig. 1A–C).

The three MC1R-derived epitopes MC1R$_{244}$, MC1R$_{283}$ and MC1R$_{291}$ are processed and presented by melanoma cells as indicated by the capacity of HLA-A2/MC1R-specific CTLs to lyse MHC class I-matched melanomas (Salazar-Onfray et al., 1997). Here, CTL lines derived from melanoma patient BL, and elicited against each MC1R-derived peptide, killed efficiently the autologous tumour BL as well as the HLA-A2$^+$MC1R$^+$ melanoma cell lines DFB, DFW and FMS (Fig. 2A–C). In contrast, the HLA-A2$^-$MC1R$^+$ melanoma cell line AK was not recognized by any of the melanoma

Table 1
Recognition of HLA-A2-restricted melanoma associated epitopes by TILs derived from melanoma patients ($n = 10$).

Cell line	MC1R			MART-1 27–35	gp100			Tyrosinase 368–376
	291–299	283–291	244–252		280–288	457–466	476–485	
TIL-1206	+	+	+	+	+	+	Nt	Nt
TIL-1233	+	–	+	+	+	–	Nt	Nt
TIL-1218	+	–	+	+	+	–	–	Nt
TIL-BL	+	–	+	–	+	+	Nt	–
TIL-0549	+	+	+	–	+	Nt	Nt	–
TIL-MR	–	–	–	+	–	–	–	–
TIL-IA	–	–	–	+	–	–	–	–
TIL-AMK	–	–	–	+	–	–	–	Nt
TIL-DL	–	–	–	–	–	–	–	–
TIL-DF	–	–	–	–	–	–	+	–

Nt: not tested.

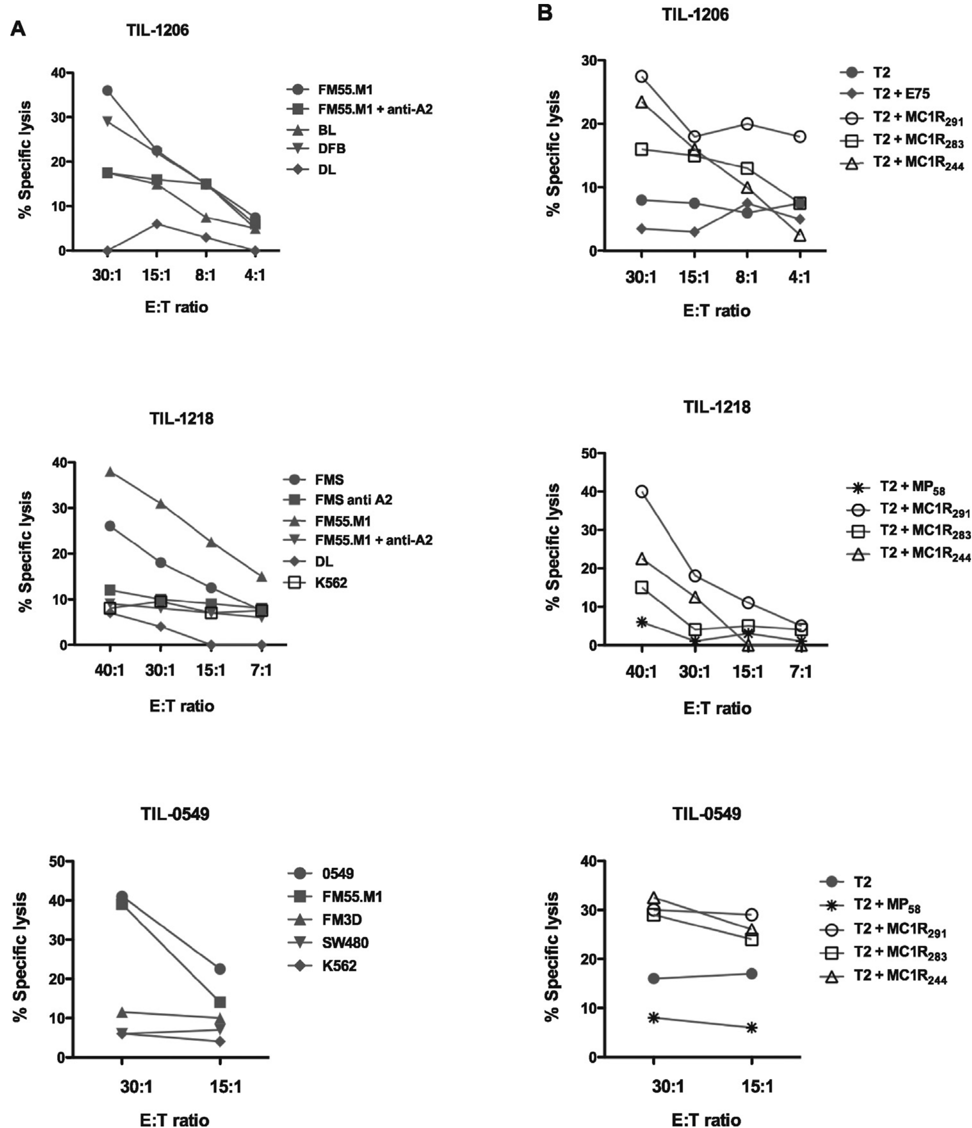

Fig. 3. Anti-melanoma TILs from melanoma patients can recognize synthetic peptides derived from MC1R. HLA-A2.1 restricted melanoma-specific TILs derived from melanoma patients BL, 1206, and 0549 were tested in a standard ^{51}Cr release assay against. (A) HLA-A2$^+$/MC1R$^+$ (FM55.M1; BL; DFB; 0549), HLA-A2$^+$MC1R$^-$ (DL; FM3D), or HLA-A2$^-$MC1R$^+$ (AA) melanoma cell lines and controls as well as against; (B) peptide-loaded T2 cells at different E:T ratios. The monoclonal antibody anti-HLA-A2 HB-54 was used to block the specific cytotoxicity. TIL-1206 recognized the two allogeneic HLA-A2$^+$MC1R$^+$ melanoma cell lines FM55.M1 and DFB. Conversely, the cytotoxic activity of TIL-1206 against FM55.M1 was blocked with an anti-HLA-A2 antibody, demonstrating HLA-A2-restriction. TIL-1206 recognized efficiently T2 target cells coated with the three MC1R epitopes and did not kill un-pulsed T2 cells or T2 cells loaded with E75 peptide. TIL-0549 recognized the autologous tumour cells as well as the allogeneic HLA-A2$^+$ melanoma cell line FM55.M1. Furthermore, TIL-0549 did not kill the HLA-A2$^+$ colon cancer cell line SW480 nor the HLA-A2$^+$/MC1R$^-$ melanoma cell line FM3D. The TIL cell line 1218 (TIL-1218) recognized the two allogeneic HLA-A2$^+$ cell lines FMS and FM55.M1. As for TIL1206, addition of an anti-HLA-A2 monoclonal antibody impaired recognition.

patient-derived HLA-A2/MC1R-specific CTL lines included in this study (Fig. 2A–C). Furthermore, the MC1R-specific CTLs did not lyse the HLA-A2$^+$MC1R$^-$ colon carcinoma SW480, or the ovarian carcinoma OVA3507 cell lines (Fig. 2A–C). Interestingly, all the tested HLA-A2$^+$ melanoma target cells were also specifically lysed by CTL lines elicited by gp100$_{280–288}$ with similar response amplitudes compared to the three MC1R-derived peptide-specific CTL lines (Fig. 2D). Conversely, a CTL line specific for HLA-A2/MP$_{58–66}$ failed to lyse the majority of the tested melanoma cell lines and displayed at most, a marginal lysis of some of the melanoma cell lines (Fig. 2E). Taken together, our results demonstrate that HLA-A2/MC1R-specific CTLs derived from PBMC of melanoma patients specifically recognized and lysed HLA-A2$^+$ target cells loaded with MC1R peptides as well as HLA-A2$^+$MC1R$^+$ melanoma cell lines, as efficiently as CTL responses to dominant HLA-A2-restricted MAA such as gp100$_{280–288}$.

The three HLA-A2-restricted MC1R-derived epitopes are recognized by TILs

The cytotoxicity of a panel of HLA-A2-restricted CTL lines derived from TILs was assessed against the three MC1R epitopes (Table 1). In principle, our results demonstrated efficient recognition of HLA-A2$^+$/MC1R$^+$ melanoma cell lines (Table 1 and Fig. 3A) and of target cells loaded with each MC1R-derived peptide (Table 1 and Fig. 3B) by half of the tested TILs. These interactions were efficiently blocked upon adding an anti-HLA-A2 monoclonal antibody, demonstrating HLA-A2-restriction (Fig. 3A). Additionally, our tested TILs did not kill HLA-A2$^-$ or HLA-A2$^+$/MC1R$^-$ cancer cell lines (Fig. 3A). For example, TIL-0549 did not kill the HLA-A2$^+$/MC1R$^-$ melanoma cell line FM3D nor the HLA-A2$^+$/MC1R$^-$ colon cancer cell line SW480 (Fig. 3A). Furthermore, TILs did not kill un-pulsed HLA-A2$^+$ target cells or HLA-A2$^+$ target cells loaded with control peptides (Fig. 3B). Interestingly, at least two of each of the three HLA-A2-restricted MC1R epitopes comprised within this study (MC1R$_{291}$ and MC1R$_{244}$) were always recognized by 50% of the analyzed TILs (Table 1 and Fig. 3B). However, it should also be noted that two TILs (1206 and 0549) efficiently killed T2 target cells coated with all three HLA-A2-restricted MC1R epitopes (Fig. 3B).

Identification of MC1R$_{244}$- and MC1R$_{291}$-specific CD8$^+$ T lymphocytes in TIL populations of melanoma biopsies

Fifteen MC1R-specific CD8$^+$ T cell clones were isolated from TIL-1206 cell line, which recognized target cells presenting the three MC1R epitopes including the allogeneic HLA-A2$^+$/MC1R$^+$ melanoma cell line FM55.M1 (Table 1 and Fig. 3A). Three of the fifteen T cell clones; N3, N4 and N9 efficiently recognized HLA-A2$^+$ T2 target cells exogenously loaded with MC1R$_{244}$ (Fig. 4). In contrast un-pulsed T2 cells or T2 cells loaded with control peptides were not recognized. The other twelve clones did not show specificity to any of the MC1R peptides (data not shown). Moreover, the presence of HLA-A2/MC1R$_{291}$-specific CD8$^+$ T cells in TIL populations from biopsies of melanoma patients was established using HLA-A2/MC1R$_{291}$ tetramers (Fig. 5). Indeed, HLA-A2/MC1R$_{291}$-specific CD8$^+$ cells were identified in TILs from three different patients (Fig. 5A). Interestingly, the frequencies of MC1R$_{291}$-positive CD8$^+$ cells in the analyzed lines were higher compared to the well-established melanoma-associated HLA-A2-restricted epitope MART-1 that is usually considered as more dominant and with higher affinity to HLA-A2 compared to the MC1R epitopes (Fig. 5B and Supplementary Figs. S1 and S2). The low solubility of MC1R$_{283}$ did not allow us to produce MHC complexes stable enough for reliable tetramerisation, preventing us from assessing the presence of HLA-A2/MC1R$_{283}$-specific CD8$^+$ TILs in melanoma biopsies. This is

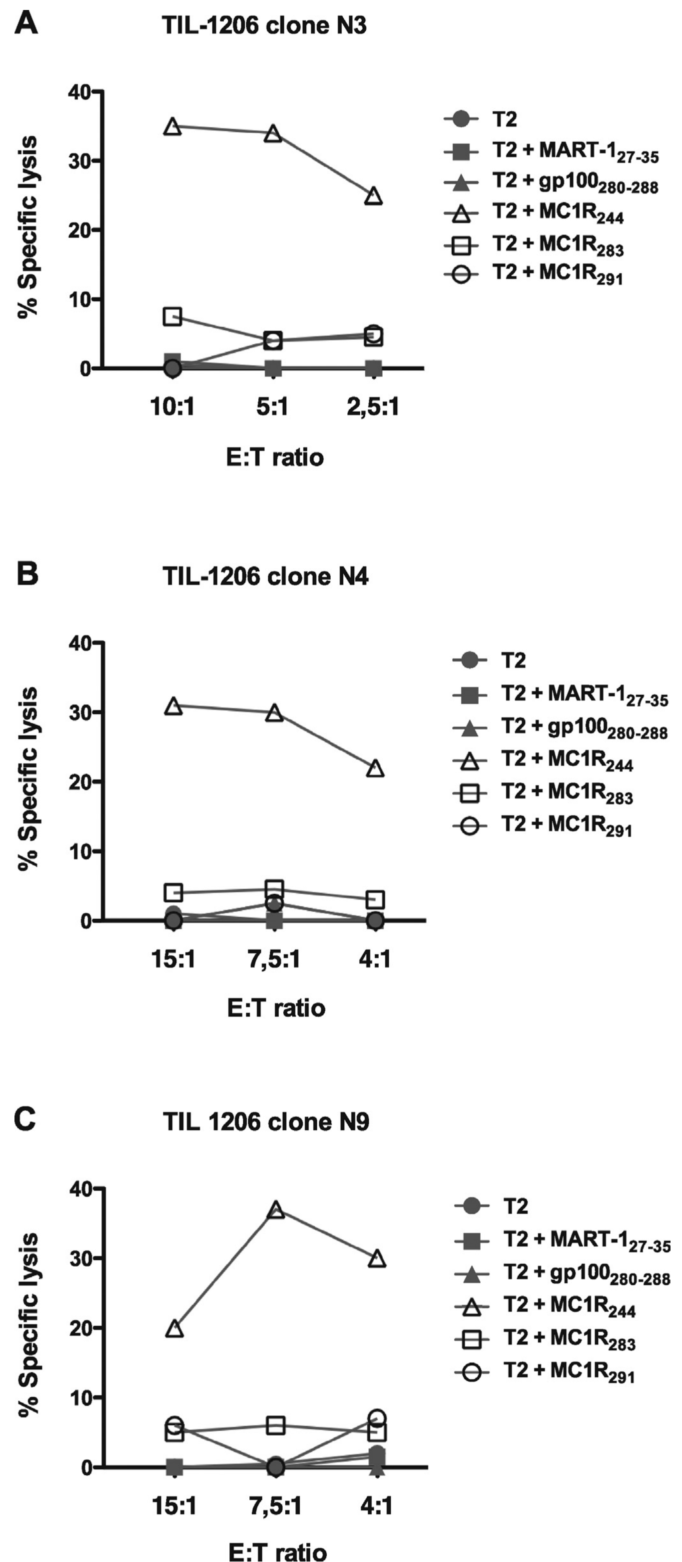

Fig. 4. Isolation of melanoma-specific CD8$^+$ T cell clones derived from TIL of the melanoma patient 1206 which recognize the MC1R$_{244}$ epitope. Clones derived from the melanoma-specific TIL line 1206 were isolated by standard limiting dilution assays as described in methods and tested against MART-1$_{27–35}$, gp100$_{280–288}$, MC1R$_{244}$, MC1R$_{283}$ and MC1R$_{291}$ peptides loaded T2 target cells in an standard ^{51}Cr release assay at different E:T ratios. Three of fifteen clones were specific for MC1R$_{244}$. (A) Clone N3. (B) Clone N4. (C) Clone N9.

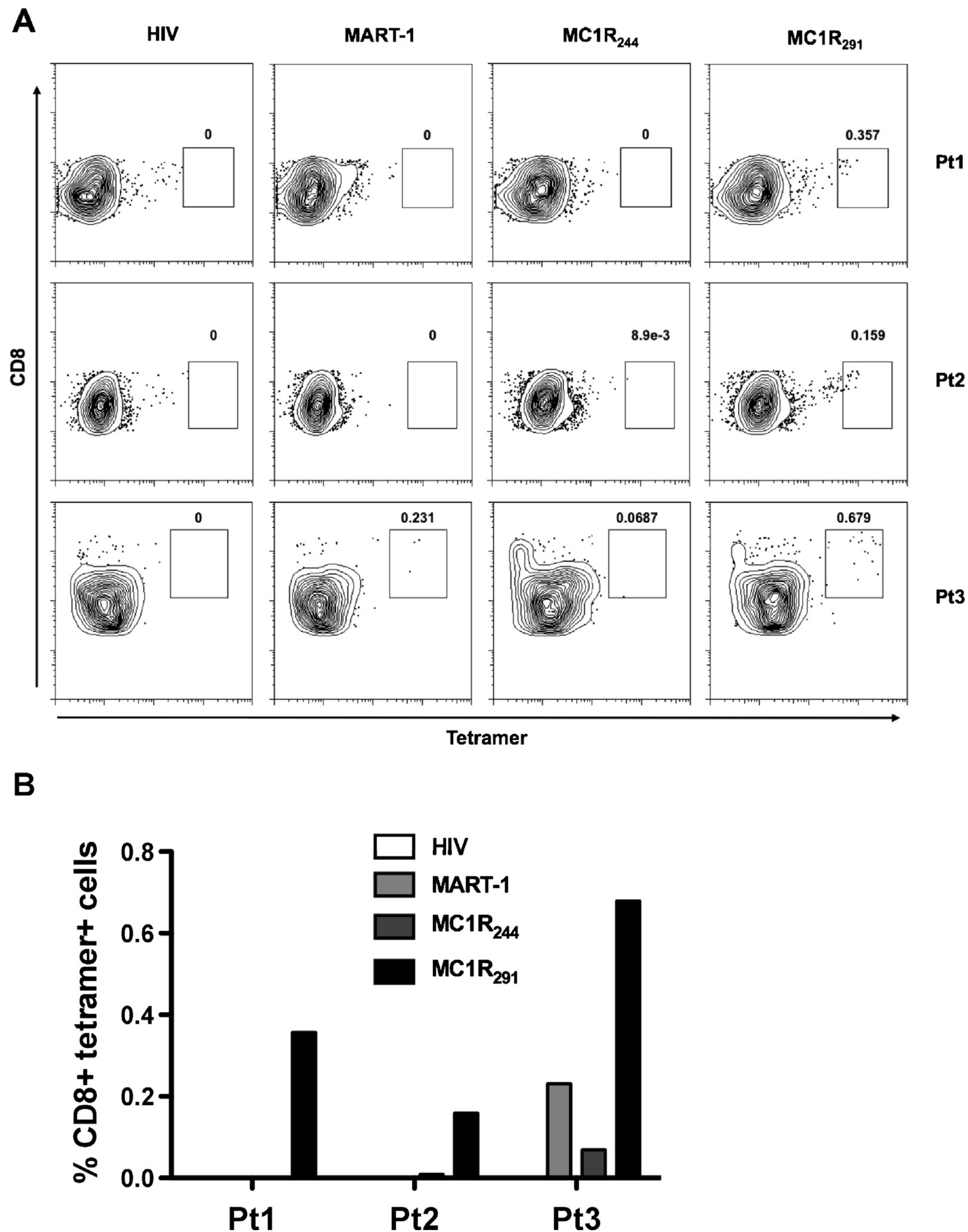

Fig. 5. Detection of MC1R-specific $CD8^+$ T cells in TILs from melanoma patients. In order to assess the presence of MC1R-specific T cells into TIL populations, HLA-A2 restricted melanoma-specific TILs derived from biopsies of three melanoma patients were stained with tetramers labelled with phycoerythrin (PE) and containing $MC1R_{244}$, $MC1R_{291}$, MART-1_{27-35}, and HIV_{77-85} peptides. Lymphocytes were gated on the basis of forward and side scatter, and the $CD8^+$ population was additionally gated. The frequency of tetramer-positive cells was determined in the $CD8^+$ cells in TILs and the gate for tetramer positive cells was placed considering HIV as a negative control. Despite the fact that HIV-specific cells can be detected using tetramers in peripheral blood of HIV seronegative individuals (Alanio et al., 2010; Legoux et al., 2010), we consider that the percentage of HIV positive cells in TILs populations isolated from melanoma metastases should be close to zero. (A) Contour plots from each analyzed patient. The numbers indicate the tetramer-positive cells as a percentage of $CD8^+$ cells. (B) Quantification of tetramer positive cells from three TILs of different melanoma patients.

also well in line with our previous observations that TILs do not recognize $MC1R_{283}$ at the same extent as $MC1R_{291}$ or $MC1R_{244}$.

Discussion

This study demonstrates the presence of HLA-A2/MC1R-specific $CD8^+$ TILs in biopsies from melanoma patients. Furthermore, TIL-derived $CD8^+$ T cell clones recognize efficiently target cells loaded with MC1R-derived peptides. We have also demonstrated the existence of MC1R-specific $CD8^+$ T cell precursors into patients' PBMC and that three MC1R-derived peptides are immunogenic. In addition, our data indicates that MC1R-derived peptides are capable to induce *in vitro* specific anti-melanoma CTLs with similar skill that well-established immunogenic cancer and viral HLA-A2-restricted peptides ($gp100_{280-288}$ and MP_{58-66} respectively). These data provide support for the potential future use of these epitopes for peptide-, DC- or T cell-based immunotherapies for combatting melanoma.

Our results also confirmed that HLA-$A2^+$ $MC1R^+$ melanoma cells present the three MC1R epitopes that we previously described (Salazar-Onfray et al., 1997), resulting in specific recognition by

both TILs and CTL lines derived from melanoma patients. Remarkably, the existence of naturally occurring anti-MC1R specificities among TIL populations demonstrates that these lymphocytes are not eliminated by selection mechanisms in the thymus. The maintenance of T cells with low reactivity to self-peptides during thymic selection has been described, providing naïve T cells with tonic TCR signals that promote long-term peripheral survival (Sprent and Surh, 2011). Despite the fact that T cells suffer a TCR desensitization process after positive selection (Sprent and Surh, 2011), high-avidity and highly proliferation-sensitive naïve CD8+ T cells against MC1R epitopes could be recruited and educated to induce potent anti-melanoma cellular immunity (Thornwall et al., 1997; Xia et al., 1996).

The protein MC1R is over-expressed in melanoma cell lines and in a majority of fresh melanoma tissues, including uveal melanoma (Lopez et al., 2007; Salazar-Onfray et al., 2002). In contrast, lower expression levels were detected in normal tissues including adrenal gland, cerebellum, placenta, liver as well as *in vitro*-stimulated monocytes/macrophages and DCs (Salazar-Onfray et al., 2002; Thornwall et al., 1997). Thus MC1R protein expression levels in different tissues may allow for differential presentation of peptide epitopes that could be recognized as anomalous by the immune system. Moreover, the potential use of the overlapping peptide epitopes concept (Melief and van der Burg, 2008) may ensure that DCs stimulate both CD4+ helper T cells and CD8+ T cells, resulting in stronger and more effective anti-tumour immune responses as previously demonstrated for P53 antigens in ovarian and colon cancer (Leffers et al., 2009; Speetjens et al., 2009).

The present study describes the generation from PBMC or the detection in TILs of HLA-A2-restricted anti-MC1R specific CTLs in advanced stage IV melanoma patients using autologous APCs loaded with three HLA-A2-restricted MC1R-derived epitopes. Moreover, the MC1R-specific CTLs derived from PBL or TIL recognized target cells loaded with the three MC1R peptides as well as MC1R+ tumour cells, demonstrating melanoma-specificity and HLA-A2 restriction. However, due to the heterogeneity of patient's HLA haplotypes, we cannot discard the presence of undetected CTLs restricted to peptides presented in the context of another isotypes. This capacity to trigger CTL responses has been previously shown for dominant epitopes, but more recently for low-affinity HLA-A2-restricted peptides (Gross et al., 2004; Kawakami et al., 1994c; Vonderheide et al., 2004).

The MC1R-derived epitopes complement previous reported screenings of the specific reactivity of anti-melanoma CTLs derived from TILs, which demonstrated the predominance of epitopes derived from MART-1 and gp100 in the context of HLA-A2 (Kawakami et al., 1994c; Sakai et al., 1997). In this regard, the use of MC1R-derived peptides could increase the overall anti-melanoma CD8+ T cell repertoire, which in turn could significantly improve the efficacy and predictability of current treatments. In fact, the presence of MC1R-specific T cell responses against melanoma has also been reported to appear after a polyvalent peptide vaccine (Reynolds et al., 1998) and others have associated vitiligo reactions with the presence of low avidity MC1R-specific CD8+ T cells in a melanoma patient (Wankowicz-Kalinska et al., 2006). Indeed, this is relevant considering the findings of Kvistborg et al. who described how melanoma-specific T-cell reactivity identified in TILs *in vitro* can predict anti-tumour reactivity and clinical efficacy post-transfer into patients (Kvistborg et al., 2012). Moreover, recent phase I and II clinical studies have obtained favourable results with a vaccination protocol that involved the administration of modified and wild type versions of the low affinity telomerase-derived $TERT_{572}$ peptide in patients with solid tumours (Mavroudis et al., 2006; Vetsika et al., 2012). In addition, advances in the design of agonistic modified peptides have demonstrated the capacity to enhance immunogenicity and induce effective anti-tumour CTL responses (van Stipdonk et al., 2009). Although these approaches are promising, it remains to be seen if modified MC1R-derived peptides can generate MC1R-specific CTL responses of higher extent compared to native epitopes.

In conclusion, our study indicates that HLA-A2-restricted MC1R-derived epitopes are useful melanoma-associated antigens recognized by CTLs and TILs derived from melanoma patients, and that they may be considered in the future development of immunotherapy against melanoma.

Conflicts of interest

No potential conflicts of interest were disclosed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2013.10.002.

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