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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 °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 °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 °C for 4 h. Then,

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