

# Melanocortin 1 Receptor Is Expressed by Uveal Malignant Melanoma and Can Be Considered a New Target for Diagnosis and Immunotherapy

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**PURPOSE.** Uveal melanoma is the most common primary malignant ocular cancer in adults. This tumor has a distinct expression pattern of markers compared with cutaneous melanoma. MC1R is under study as a potential target for antitumor immunity. Because of the potential immunogenicity of MC1R, it is important to evaluate its expression on uveal melanomas.

**METHODS.** Two novel monoclonal antibodies (MP1.1C11 and MP1.1B7) were used to examine the expression of MC1R in uveal melanomas. Tissue samples obtained from 17 patients were analyzed for expression of MC1R by immunohistochemistry. Additionally, uveal melanoma cell lines were treated with proinflammatory cytokines, after which MC1R cell surface expression was analyzed by flow cytometry.

**RESULTS.** Results demonstrated that MC1R is expressed by uveal melanoma to a significantly greater extent than other melanoma markers. With the use of MP1.1C11 or MP1.1B7, MC1R was detected in 95% of the tested melanoma tissues, including one liver metastasis. In contrast, MART-1, S100-specific protein, and gp-100 were only expressed by 66%, 33%, and 67% of the analyzed samples, respectively. Results also demonstrated that even though MC1R is mainly located intracellularly, its cell surface expression can be promoted by cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-10.

**CONCLUSIONS.** These observations support the inclusion of MC1R in the panel of markers for the diagnosis of uveal melanoma. Therapeutic use of MC1R-specific antibodies targeting cytokine-induced MC1R potentially requires expression of the target molecule on the surfaces of tumor cells. Data presented here support MC1R as a new marker and a putative therapeutic target for uveal melanoma. (*Invest Ophthalmol Vis Sci.* 2007; 48:1219–1227) DOI:10.1167/iov.06-0090

Uveal melanoma, arising from the choroid or the ciliary body of the eye, is a highly malignant tumor. Although it has a relatively low incidence (six cases per 1,000,000 per year), this type of cancer is the most common primary ocular malignancy in adults.<sup>1</sup> Although the location of this tumor may lead to early diagnosis and effective treatment of the primary lesion by enucleation, radiotherapy, local resection, and transpupillary thermotherapy,<sup>2</sup> these treatments do not prevent the development of metastases. One of the characteristics of uveal melanoma is hematogenic spread to the liver, associated with up to 50% of patient deaths.<sup>3</sup> In fact, surgical removal of liver metastases is possible for few patients<sup>4</sup> because uveal melanoma metastases are highly resistant to traditional chemotherapy.<sup>2,3</sup> Several immunotherapeutic strategies are under consideration for the treatment of cutaneous melanoma in patients with advanced metastatic disease.<sup>5–7</sup> In contrast, equivalent therapeutic approaches for uveal melanoma are unavailable, which emphasizes the importance of research oriented to the identification of specific antigens for this tumor.

Furthermore, because of heterogeneous morphology, immunohistochemical confirmation is sometimes required to rule out nonmalignant ocular pigmentary diseases.<sup>8,9</sup> Although uveal melanoma expresses some markers of cutaneous melanoma, the expression of these proteins is restricted to a fraction of tumor tissues.<sup>10–12</sup> Monoclonal antibodies most commonly used to diagnose uveal melanoma are S-100, which is specific for a protein derived from bovine brain cross-reacting with melanoma and melanocytes,<sup>13</sup> HBM45, which is specific for the melanosomal gp100 protein,<sup>14</sup> and A103, which recognizes the Melan-A/Mart-1 protein.<sup>11</sup> However, mutations or loss of expression resulting from immunologic selection can restrict the use of these markers as a consistent diagnostic tool. Consequently, more accurate diagnosis of uveal melanoma requires markers that are reliably expressed by this type of tumor. With the use of a panel of monoclonal antibodies, we recently characterized the tissue distribution of the melanoma marker MC1R in normal and tumor tissues.<sup>15</sup> MC1R corresponds to the receptor for the  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH), a tridecapeptide derived from the precursor molecule proopiomelanocortin (POMC).<sup>16</sup> This molecule is primarily released by the pituitary but is also released by immunocompetent cells.<sup>17,18</sup>  $\alpha$ -MSH is a potent stimulator of the pigmentation and differentiation of pigmented cells, including melanoma cells.<sup>19</sup> Five different subtypes of melano-

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cortin receptors with different tissue distribution have been described in humans.<sup>20,21</sup> Among these five subtypes, MC1R was originally shown to be predominantly expressed by melanoma and melanocytes.<sup>19,22</sup> In addition, we have described that high levels of MC1R are expressed on cell lines derived from primary and metastatic cutaneous melanoma.<sup>15</sup> Nonetheless, it was subsequently observed that MC1R can also be expressed at low levels by other tissues and cells, including human testis, ovary, adrenal gland, keratinocytes, dendritic cells, and activated monocytes.<sup>15,18,23,24</sup> This study represents the first report on the analysis of MC1R expression by uveal melanoma.

Previously, we demonstrated that three MC1R-derived HLA-A2 nonameric peptides can induce the expansion of peptide-specific CTLs from peripheral blood mononuclear cells (PBMCs) of healthy HLA-A2<sup>+</sup> donors. These peptide-specific CTLs were also able to recognize HLA-A2<sup>+</sup> melanoma cells expressing MC1R, demonstrating that the MC1R-derived peptides are naturally processed and presented by major histocompatibility complex (MHC) class I on the surfaces of melanoma cells.<sup>25</sup> Based on these observations, MC1R could be considered an immunologic target in humans, which gives rise to the possibility of antigen-specific immunotherapy. Active immunotherapy based on MC1R-specific T cells or vaccination with antigen-presenting cells loaded with MC1R-derived peptides may constitute a valid alternative for melanoma treatment because it has been demonstrated for other melanoma-associated antigens.<sup>5-7</sup>

Furthermore, the success of a potential antibody-based immunotherapy targeting MC1R would depend on the differential cell surface expression of MC1R in melanoma cells compared with healthy tissues. Our previous studies demonstrated that MC1R is predominantly expressed intracellularly in cutaneous melanoma.<sup>15</sup> In addition, surface expression can be induced on immunocompetent cells such as monocytes and macrophages by several cytokines, as demonstrated by other investigators and us.<sup>15,26</sup> However, the extent to which MC1R could be induced on the melanoma cell surface has not been established. Because MC1R surface expression may be important, particularly for potential antibody-mediated therapy, we have characterized the upregulation of MC1R surface expression on uveal melanoma cell lines using proinflammatory cytokines including IFN- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-10. Our data provide new evidence in support of MC1R as a novel, highly specific marker for uveal melanoma and suggest ways of enhancing the efficiency of MC1R-specific immunotherapy.

## MATERIALS AND METHODS

### Cell Lines

OCMS1 and OCMS3 ocular melanoma lines were kindly provided by Dr. M. Jager (University of Leiden, The Netherlands).<sup>27</sup> C1R-A2 is a lymphoblastoid cell line that does not express MC1R.<sup>15</sup> FM55, a cutaneous melanoma cell line, was kindly provided by Jesper Zeuthen (Cancer Society, Copenhagen, Denmark). The OCMS4, OCMS5, OCMS6, and OCMS7 uveal melanoma cell lines were established in our laboratory from enucleated tissues obtained from the Department of Ophthalmology (Hospital del Salvador, Santiago, Chile). Tissue samples were acquired after informed consent and with the approval of the ethics committee (Bioethical Committee for Human Research of the Faculty of Medicine, University of Chile) and the regulatory authorities, during routine surgical management of patients. Briefly, fresh melanoma tissues obtained after enucleation were washed in phosphate-buffered saline (PBS) and mechanically homogenized. Cells were then counted and cultured in RPMI 1640 (Invitrogen Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) in the presence of 1  $\mu$ g/mL cyclosporin A (Sigma Aldrich, St. Louis, MO)

for 48 hours, and expanded for at least 4 weeks before phenotypic characterization. All procedures were performed in adherence to the tenets of the Declaration of Helsinki.

### FACS Flow Cytometry Analysis of MC1R Surface and Intracellular Expression

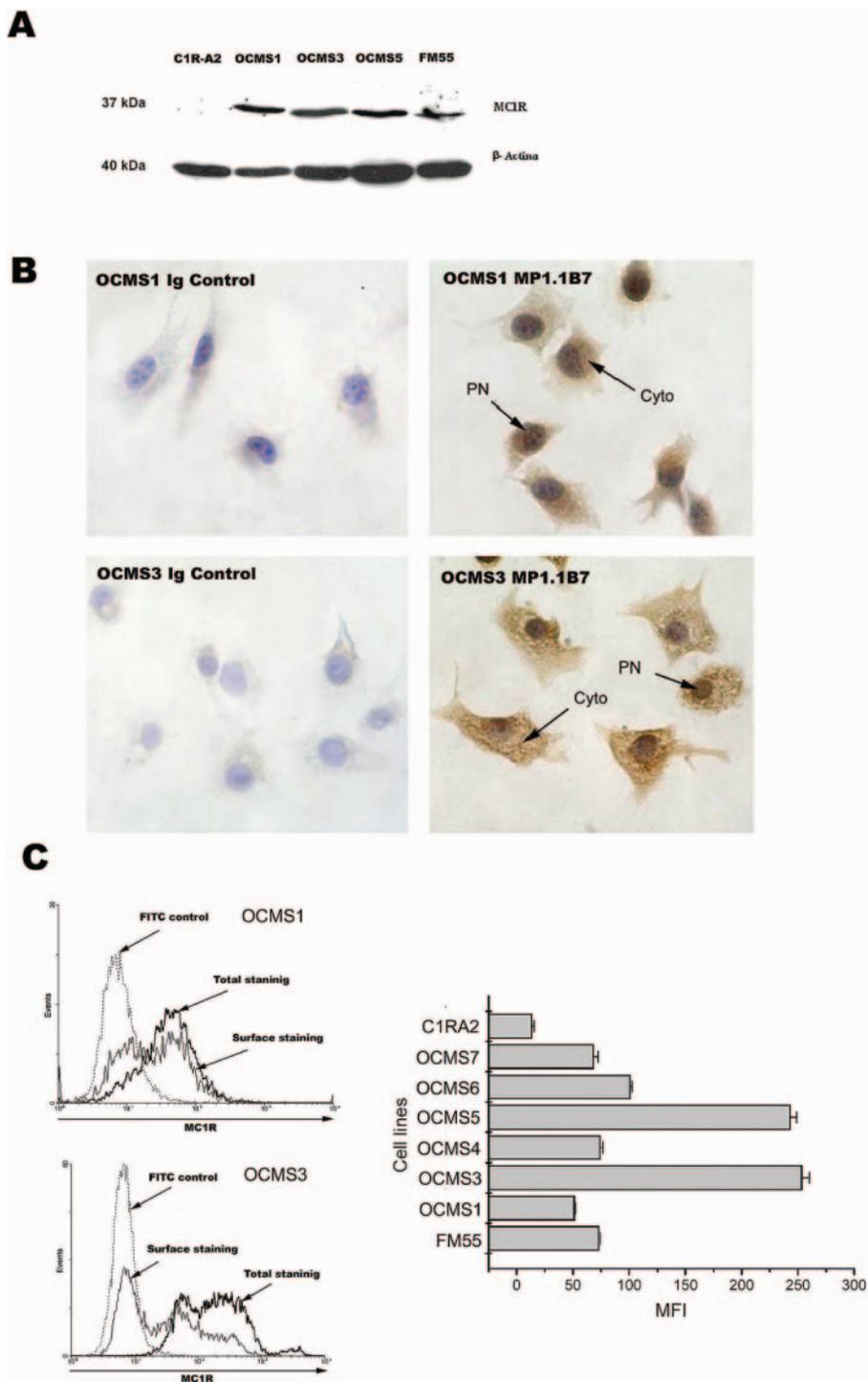
Anti-MC1R mAbs MP1.1B7 and MP1.1C11, previously described as restricted to the extracellular domain of MC1R, were kindly provided by Vijay Chhajlani (Lead Discovery Department, AstraZeneca, Wilmington, DE). Cultured uveal melanoma cell lines were washed three times with cold PBS, tested for cell viability by trypan blue exclusion, and prefixed by 10-minute incubation with 0.5% paraformaldehyde in PBS. Cells either were washed in PBS for 10 minutes on ice or were permeabilized with 2% digitonin (Sigma). Cells ( $3 \times 10^5$  /well) were added to a 96 V-bottom well and incubated with 2  $\mu$ g/mL MC1R-specific mAb (MP1.1B7 or MP1.1C11)<sup>15</sup> or control IgG1 mAb for 30 minutes on ice. After staining, cells were washed twice with PBS and incubated with a secondary rabbit anti-mouse immunoglobulin FITC-conjugated antibody (2  $\mu$ g/mL; Dako, Glostrup, Denmark) for another 30 minutes on ice. After incubation, the cells were washed three times with PBS 0.01% Tween 20 (PBS-Tween) and were fixed again with 1% paraformaldehyde in PBS containing 0.1% FCS and kept at 4°C until analysis by FACS (BD Biosciences, San Diego, CA).

### Western Blot

Cell pellets from cell lines and fresh tissues ( $5 \times 10^6$  cells) were suspended in ice-cold lysis buffer (0.5% Triton X-100, 50 mM Tris-HCl, 300 mM NaCl, 10  $\mu$ g/mL aprotinin, 10  $\mu$ g/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride) for 30 minutes at 4°C and centrifuged at 14,000g for 20 minutes. The supernatant was mixed with SDS-PAGE sample buffer and then separated on 12.0% SDS-polyacrylamide gels. For immunoblots, proteins were electrotransferred onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore Corp., Bedford, MA). Membranes were blocked for nonspecific antibody binding with Tris-buffered saline containing 5% BSA. Subsequent immunostaining steps were performed in phosphate-buffered saline with 0.1% PBS-Tween at room temperature. Membranes were then incubated with the primary antibody (3  $\mu$ g/mL; MP1.1B7) for 1 hour and then with a horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin as a secondary antibody (2  $\mu$ g/mL; Amersham, Buckinghamshire, UK) for 30 minutes. As an internal control, a commercial polyclonal antibody against  $\beta$ -actin (2  $\mu$ g/mL; Sigma) was used to restain the membranes after stripping. Membranes were then washed in PBS-Tween four times and developed with an enhanced chemiluminescence (ECL) system (Amersham).

### Immunohistochemical Analysis

Immunohistochemical staining was performed using the standard avidin-biotin complex (ABC) technique (LSAB kit; Dako). Paraffin sections corresponding to primary ocular melanoma ( $n = 17$ ), metastatic uveal melanoma ( $n = 1$ ), cutaneous melanoma ( $n = 1$ ), or pigmented nevi ( $n = 3$ ; Department of Pathology, University of Chile Clinical Hospital) were deparaffinized and rehydrated. Tissues were depigmented with KMnO<sub>4</sub> for 5 minutes at 60°C with a microwave and whitened with oxalic acid 1%. Sections were blocked with normal horse serum for 30 minutes, excess serum was drained, and the sections were incubated with the primary antibody mouse mAb MP1.1C11 anti-MC1R and with the commercial rabbit polyclonal Ab anti-S100, mouse mAb HMB-45, and mouse mAb Melan-A (Dako) at 10  $\mu$ g/mL. All incubations were performed overnight at 8°C. Biotinylated anti-mouse IgG or biotinylated anti-rabbit immunoglobulin (for S100 staining) was used as secondary antibody, followed by ABC. The peroxidase reaction was developed using 0.6 mg/mL 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB) with 0.03% hydrogen peroxide for 6 minutes. Counterstaining was not performed. Phosphate-buffered saline (pH 7.6) was used as a rinsing agent between the different steps. For immunocytochemical analysis, the cells were cultured on glass coverslips for 2



**FIGURE 1.** MC1R is expressed in ocular melanoma cell lines. (A) Immunoblotting for MC1R shows a 37-kDa band for uveal melanoma cell lines (OCMS1, OCMS3, and OCMS5) and cutaneous melanoma cells (FM55). No band is seen for the LCL cell line C1R-A2 used as a negative control.  $\beta$ -actin was used as an internal loading control. (B) Immunocytochemical staining shows the expression of MC1R (mAb MP1.1B7) in ocular melanoma cell lines (OCMS1, OCMS3). mAb IgG was used as a negative control. (C) Ocular melanoma cell lines (OCMS1, OCMS3-OCMS7) and the cutaneous melanoma cell line FM55 were permeabilized and then stained with mAb MP1.1C11 (anti-MC1R), and MC1R mean fluorescence intensity (MFI) of expression in arbitrary units was analyzed by flow cytometry. C1R-A2 LCL cell line and ocular melanoma cell lines incubated with secondary FITC-conjugated antibody were used as a negative control.

hours at 37°C, fixed with cold methanol, and stained as described. MC1R protein expression was evaluated according to an arbitrary scale (-,  $\pm$ , +) on the basis of the immune reaction intensity in a double-blind procedure.

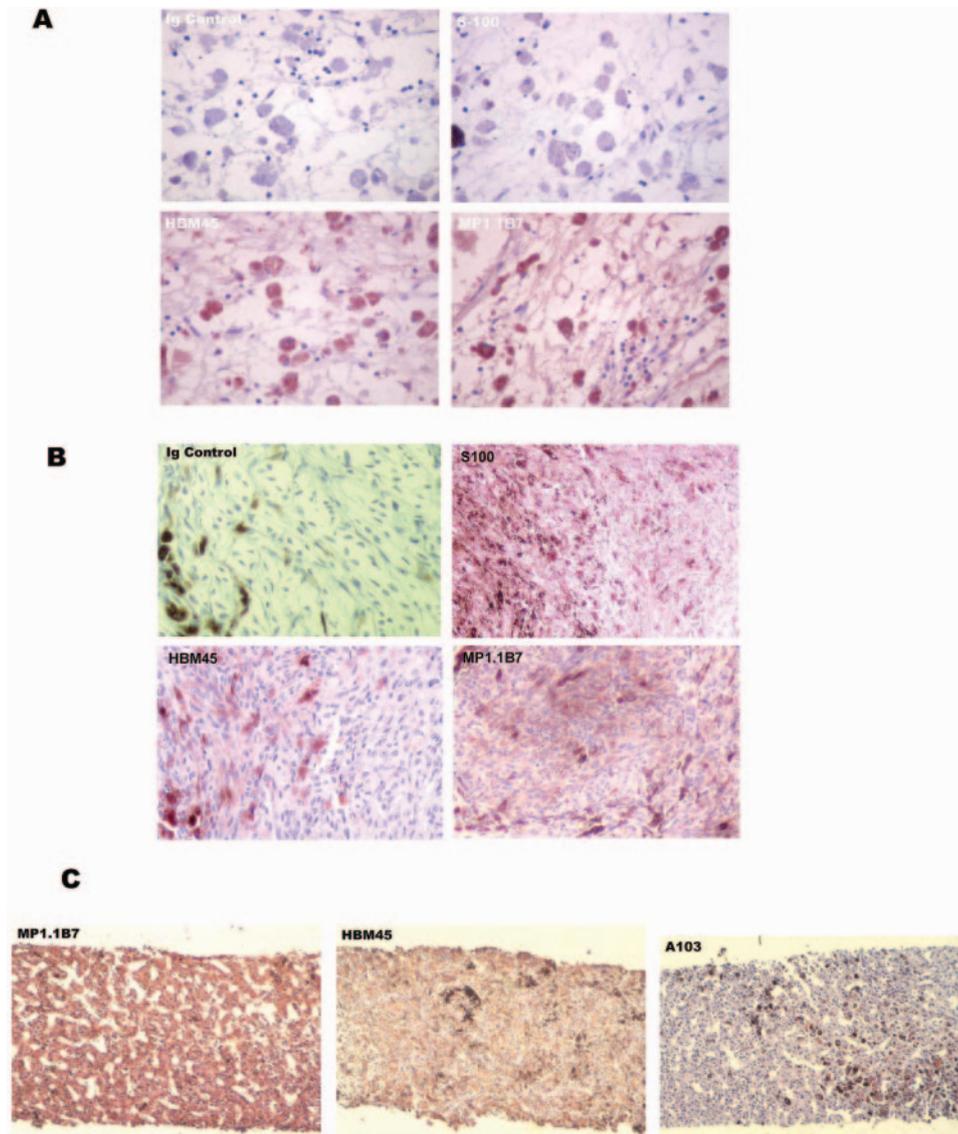
**Immunofluorescence Staining**

Uveal melanoma cells were cultured on glass coverslips for 2 hours at 37°C and fixed for 10 minutes with cold 70% methanol. Cells were then incubated with 5% BSA-phosphate buffer for 1 hour to block nonspecific antibody binding and were incubated with the MC1R-specific antibody in 1% BSA-phosphate buffer in a humid chamber at 4°C overnight. MC1R expression was visualized by incubating the cells

for 1 hour with a PE-conjugated anti-mouse immunoglobulin secondary antibody diluted in 1% BSA-phosphate buffer. As a positive control, an FITC-conjugated anti-MHC class I antibody (W6/32; eBioscience, San Diego, CA) was tested in parallel. Cells were analyzed by confocal laser scanning microscopy (LSM 510; Carl Zeiss MicroImaging, Inc., Oberkochen, Germany).

**Cytokine and  $\alpha$ -MSH Treatment of Ocular Melanoma Cells**

Ocular melanoma cells ( $1 \times 10^6$ /mL) were incubated in 2 mL RPMI 5% FBS in a 24-well plate in the presence or absence of IFN- $\gamma$  (500 U/mL;



**FIGURE 2.** Anti-MC1R antibody homogeneously stains primary and liver metastatic uveal melanoma tissues with higher intensity and specificity than standard melanoma-specific antibodies. Series of primary uveal melanoma paraffin sections of (A) OCM 3802 and (B) OCM 3784 were immunostained with an IgG control mAb, with S-100, HBM45-specific melanoma mAbs, and with MP1.1B7 mAb directed against MC1R. (C) Liver metastatic uveal melanoma tissue was stained with mAb A103 (anti-Mart-1 protein), mAb HBM45 (anti-gp100 protein), and mAb MP1.1B7 (anti-MC1R protein). The MP1.1B7 mAb exhibited stronger staining than the other tested mAbs. Nuclear staining was performed using hematoxylin solution.

Boehringer Ingelheim, Ingelheim, Germany), TNF- $\alpha$  (1 ng/mL; US Biological, Swampscott, MA), IL-4 (500 U/mL; US Biological), IL-10 (50 U/mL; US Biological), or the MSH analogue Nle,<sup>2</sup> D-Phe<sup>7</sup>- $\alpha$ -MSH ( $10^{-9}$  M; Bachem, Heidelberg, Germany). Cells were analyzed by FACS after overnight incubation. In some experiments, melanoma cells were incubated with 0, 50, 100, and 500 U/mL IFN- $\gamma$  for 2, 4, 8, 12, and 24 hours. Viability of cytokine-treated cells was checked by trypan blue exclusion.

### Statistical Analysis

Experiments were repeated at least twice to confirm the consistency of the data. Experiments involving cytokine treatment of melanoma cells were performed in triplicate to obtain the mean, SD, and SE. Differences among groups were calculated by the Student *t* test with a software program (Origin; RockWare Inc., Golden, CO).  $P > 0.05$  was considered statistically significant.

## RESULTS

### Expression and Cellular Location of MC1R in Uveal Melanoma Cell Lines

Specificity of the anti-MC1R mAbs MP1.1B7 and MP1.1C11 has been described as restricted to the extracellular domain of

MC1R and is expressed on cutaneous melanoma cell lines and tissues.<sup>15</sup> These antibodies were used to examine one fresh uveal melanoma tissue (OCMS5) and two uveal melanoma cell lines (OCMS1 and OCMS3; Fig. 1A). Both mAbs showed similar activities and were therefore used interchangeably in this study. As a negative control, the lymphoblastoid cell line (C1R-A2), which does not express MC1R,<sup>15</sup> was used. In addition, a cutaneous melanoma cell line, FM55, was used as a positive control. All melanoma samples were positive for a 37-kDa band, which matches the predicted size for the MC1R protein. As expected, this band was absent in lysates of the C1R-A2 cell line (Fig. 1A). MC1R expression on uveal melanoma cell lines was confirmed by immunohistochemistry. In fact, OCMS1 and OCMS3 melanoma cell lines were intensely stained by MP1.1B7 antibody but not by an IgG control mAb (Fig. 1B). OCMS1 and OCMS3 showed heterogeneous surface expression of MC1R. The intracellular location of MC1R was confirmed through cell permeabilization and flow cytometry analysis, which showed more intense and homogeneous staining compared with the surface staining (Fig. 1C histograms), indicating that MC1R accumulates intracellularly in uveal melanoma cells, as previously observed in cutaneous melanoma cell lines.<sup>15</sup> Strong MC1R expression was also observed in the six uveal melanoma cell lines tested (Fig. 1C)—OCMS1, OCMS3, OCMS4, OCMS5,

**TABLE 1.** Expression of MC1R (mAb MP1.1B7) and Other Melanoma Markers (mAb S100, mAb HBM45) in Human Uveal Melanoma Tissues

Uveal Melanoma	S100	Hbm45	Mp1.1b7
OCM 2767	—	+	+
OCM 2892	—	—	+
OCM 3841	—	+	+/-
OCM 3802	—	+	+
OCM 3795	—	ND	+
OCM 1260	—	—	+
OCM 2736	+	ND	+
OCM 2862	—	ND	+
OCM 3054	+	+	+
OCM 3469	—	—	+
OCM 3839	—	+	+
OCM 3784	+	+	+
OCM 2858	+	+	+
OCM 2040	+	+	+
OCM 3715	—	+/-	+
OCM 2890	ND	ND	+
OCM 2889	ND	ND	+
Cutaneous primary melanoma 5530	+	+	+
Pigmented nevus 6643	—	—	—
Pigmented nevus 2815	—	—	—

ND, not determined.

OCMS6, OCMS7—most of which were established in our laboratory. MC1R located predominantly in an intracellular compartment, and the level of expression was similar to or higher than that observed for the cutaneous melanoma cell line FM55 (Fig. 1B).

### Expression of MC1R in Uveal Tissues

To determine the usefulness of available MC1R-specific antibodies for the phenotypic characterization of uveal melanoma, we performed immunohistochemical analyses of MC1R expression in a panel of paraffin sections of uveal melanoma and nonmalignant nevi. Intense, specific immunostaining for MC1R was revealed in most of the primary uveal melanoma tissues tested, as exemplified by tissues derived from patients OCM 3802 (Fig. 2A) and OCM 3784 (Fig. 2B). In total, tissues from 16 of 17 (94%) uveal melanoma patients were strongly stained by the MC1R-specific mAb MP1.1C11 (Table 1). Only one tissue sample showed a weak but still significant level of MC1R expression. In contrast, only 5 of 15 (33%) and 8 of 12 (66%) uveal melanoma tissues were positive for the S-100 mAb and the HBM45 mAb, respectively (Table 1). Although two normal nevi were negative for the three antibodies, a cutaneous melanoma showed immunoreactivity with all tested mAbs (Table 1). Furthermore, the MC1R-specific antibody showed equal or better staining on uveal melanomas (6 of 6 samples) than did the A103 mAb, which is specific for the MART-1/Melan-A protein (4 of 6 samples; Table 2). MC1R expression was homogeneous in the uveal melanoma samples analyzed. Most of these tissues showed staining that was significantly more intense for MC1R than for any of the other markers studied (Figs. 2A, 2B). In contrast, no significant staining for MC1R was observed in adjacent normal tissues, including connective and epithelial tissues.

Furthermore, we observed that MC1R was also expressed in liver metastases of uveal melanomas (Fig. 2C). Samples of metastatic tissues were stained with the MC1R-specific antibody MP1.1B7. As a comparison, the same tissue samples were stained with the two melanoma-specific antibodies HBM45 and A103. The strongest and most homogenous staining was observed for samples stained with the MC1R-specific antibody (Fig. 2C) compared with the other tested antibodies directed to melanosomal proteins (Fig. 2C).

### Cytokine Modulation of MC1R Cellular Location

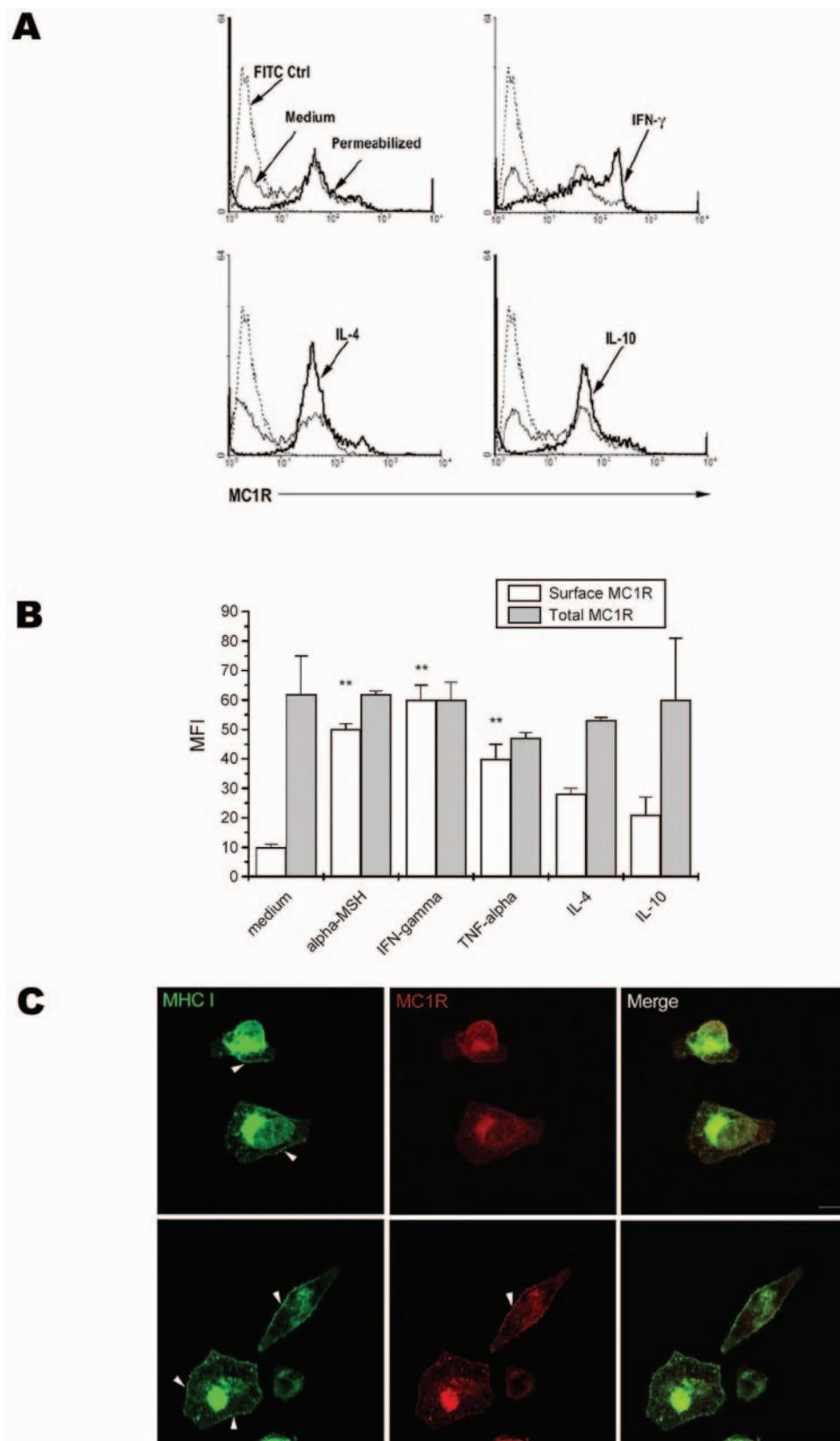
To evaluate whether the surface expression of MC1R can be modified by proinflammatory cytokines, we treated two uveal melanoma cell lines, OCMS1 and OCMS3, with IFN- $\gamma$ , TNF- $\alpha$ , IL-4, or IL-10. After cytokine treatment, MC1R surface expression was analyzed by flow cytometry, as described in Materials and Methods. In these two uveal melanoma cell lines, we consistently observed an enhanced surface expression of MC1R after cytokine treatment (Figs. 3A, 3B). In particular, treatment of ocular melanoma cells with  $\alpha$ -MSH hormone, IFN- $\gamma$ , or TNF- $\alpha$  augmented MC1R surface expression in a statistically significant manner (Fig. 3B). However, the total cellular expression of MC1R was not modified (Fig. 3B), as demonstrated by flow cytometry analyses on permeabilized cells. This impression was supported by immunofluorescence experiments on OCMS1 cells showing that in the absence of cytokine treatment, MC1R staining locates mainly at the perinuclear region and partially in the rest of the cytosol (Fig. 3C, upper center panel). In contrast, treatment with IFN- $\gamma$  resulted in a change in the location of MC1R staining, which now was also found at the cell membrane (Fig. 3C, low center panel). As a control, MHC class I surface expression was upregulated by IFN- $\gamma$ , as expected (Fig. 3C, green labeling). These results suggest that the observed increase on surface MC1R expression in response to cytokine treatment was the result of a posttranslational regulatory mechanism. To determine the dose dependency and kinetics of IFN- $\gamma$ -mediated induction of MC1R surface expression, OCMS1 cells were treated with different doses of IFN- $\gamma$  and tested by flow cytometry for MC1R expression at different time points. We observed that MC1R surface expression on treated melanoma cells was augmented in a dose-dependent manner in response to IFN- $\gamma$  (Fig. 4A). MC1R surface levels reached maximal expression at 500 U/mL of the cytokine (Fig. 4A). Additionally, the highest mobilization of MC1R to cell membrane occurred after 4 hours of incubation with IFN- $\gamma$  and declined to basal levels after 24 hours, indicating that the induction was transient (Fig. 4B).

### DISCUSSION

In this study, we provide important new evidence supporting the inclusion of MC1R as a specific molecular marker for uveal melanoma. In addition, our findings that expression of this molecule is highly restricted to tumor cells would encourage MC1R for appraisal as a potential new target for immunotherapy. Our data indicated that MC1R was abundantly expressed by most ocular melanoma cell lines studied, as shown by flow cytometry analysis of a large panel of lines. Furthermore, immunohistochemistry and Western blot analyses demonstrated MC1R expression in most samples of freshly isolated, noncultured, primary and metastatic uveal melanomas. Detection of MC1R with mAb MP1.1C11 showed sensitivity and specificity similar to or better than

**TABLE 2.** Comparison between MC1R (mAb MP1.1B7) and Melan-A-Specific Antibody (mAb A103) in Primary and Liver Metastatic Uveal Melanoma Tissues

Uveal Melanoma	A103	MP1.1B7
OCM 2767	+	+
OCM 2892	+	+
OCM 3839	+	+
OCM 2858	—	+
OCM 3715	+	+
Liver metastasis	—	+
Pigmented nevus 23784	—	—

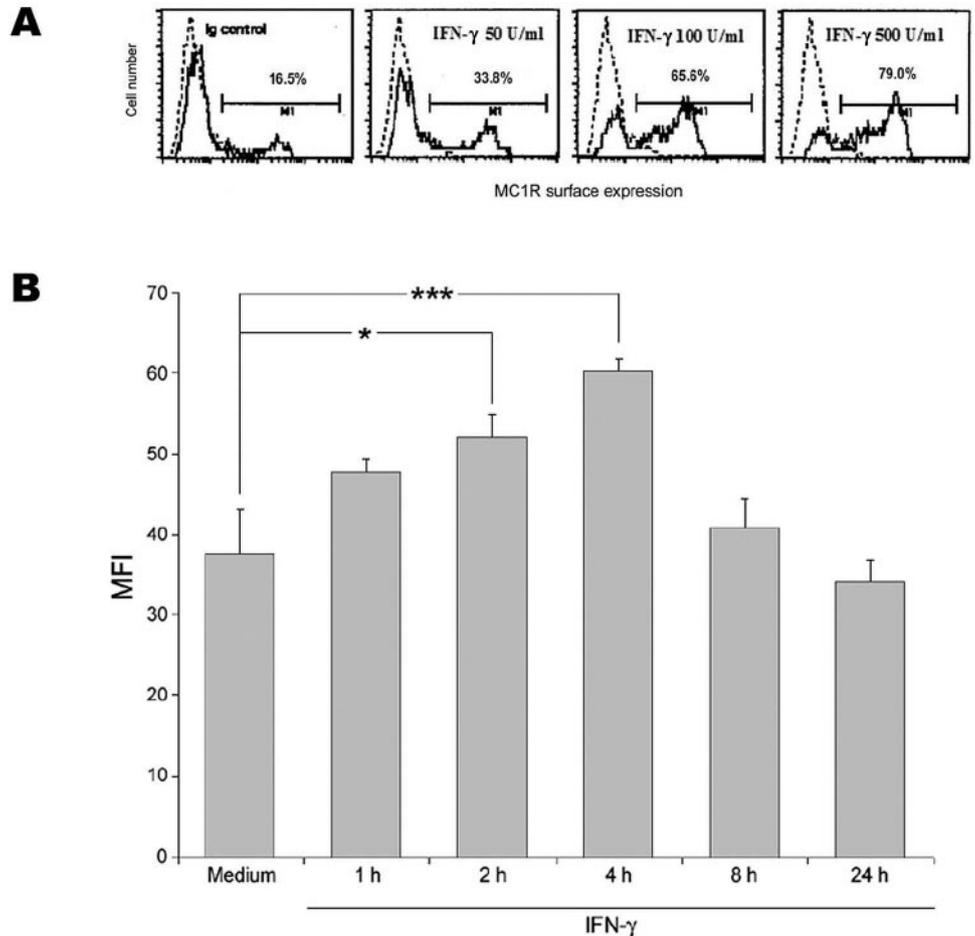


**FIGURE 3.** MC1R surface expression on ocular melanoma cell lines is enhanced by cytokines. OCMS1 cells were incubated in the presence or absence of cytokines IFN- $\gamma$  (500 U/mL), TNF- $\alpha$  (1 ng/mL), IL-4 (500 U/mL), and IL-10 (50 U/mL) or the hormone analogue Nle2, D-Phe7- $\alpha$ -MSH ( $10^{-9}$  M), known to upregulate MC1R expression. Treated cells were stained with mAb MP1.1C11 (anti-MC1R) and then analyzed by FACS. **(A)** MC1R surface expression increased in cytokine pretreated nonpermeabilized ocular melanoma cells. **(B)** Cytokine pretreatment increased MC1R surface expression in ocular melanoma cells (*white bars*) but did not alter total MC1R expression (*gray bars*). MFI, mean fluorescence intensity in arbitrary units. \*\*  $P < 0.05$  according to Student *t* test. **(C)** OCMS1 cells untreated (*upper*) or treated with 500 U/mL IFN- $\gamma$  (*lower*) were stained with mAb MP1.1C11 (anti-MC1R) followed by a PE-conjugated anti-immunoglobulin mAb or with a FITC-conjugated anti-MHC class I mAb as a positive control and then analyzed by confocal microscopy. *Arrows*: Protein surface accumulation. Results are representative of two different experiments.

those of other commonly used antimelanoma antibodies, such as S-100, A103, and HBM45.<sup>10-14</sup> Finally, we established that surface expression of MC1R in ocular melanoma cell lines can be upregulated by proinflammatory cytokines,

which may be important for the design of antibody-mediated immunotherapy.

Early reports using the binding of radiolabeled peptides<sup>20,22</sup> demonstrated that the MC1R protein is present on the surfaces



**FIGURE 4.** Dose dependency and time kinetics of MC1R IFN- $\gamma$ -induced surface expression on ocular melanoma cells. **(A)** OCMS1 cells were incubated overnight with medium only or with 50, 100, and 500 U/mL IFN- $\gamma$  stained with anti-MC1R mAb (MP1.1C11) and then analyzed by FACS. A dose-dependent increase of MC1R surface expression is observed with augmented IFN- $\gamma$  concentration. **(B)** OCMS1 cells were incubated with or without 500 U/mL IFN- $\gamma$  for 1, 2, 4, 8, and 24 hours, and then the level of MC1R expression (MFI in arbitrary units) was analyzed by FACS using mAb MP1.1C11. The highest level of expression was observed after 4-hour treatment. \* $P < 0.05$  and \*\*\* $P < 0.0005$ ; Student  $t$  test. The graph depicts results of three independent experiments performed in duplicate.

of melanoma cells. Messenger RNA encoding for MC1R could also be detected in melanoma cells and in normal melanocytes.<sup>19,26</sup> In previous studies, we found that MC1R is overexpressed in most fresh melanoma tissue and melanoma cell lines but not in carcinoma lines or LCL.<sup>15</sup> Furthermore, we found homogeneous staining of MC1R in cutaneous melanoma metastasis at various locations, indicating that this protein did not decrease its expression during tumor progression, though we were unable to detect MC1R in normal nevi.<sup>15</sup>

Uveal melanoma has a different expression pattern of surface markers than cutaneous melanoma.<sup>10-13</sup> These tumors express a number of melanoma-associated markers useful for the diagnosis of tumor lesions.<sup>10-14</sup> Because of its heterogeneous morphology, immunohistochemical analysis is sometimes necessary to confirm a diagnosis of uveal melanoma and to rule out pseudomelanoma such as choroidal nevus, peripheral exudative hemorrhagic chorioretinopathy, congenital hypertrophy of the retinal pigment epithelium, circumscribed choroidal hemangioma, and age-related macular degeneration.<sup>9,10</sup> However, because the specificity and sensitivity of the different antibodies are variable and because melanoma-associated proteins have differential expression in distinct lesions as a result of mutations and immunologic pressure, new markers are necessary for a more accurate cell characterization. Furthermore, immunotherapy requires a broadly expressed antigenic target to validate massive use in patients. In this study, we compared MC1R-specific mAbs with other melanoma-specific mAbs previously used in uveal melanoma diagnosis. We demonstrated that although several melanoma samples were positive for all used specific mAbs (S-100, HBM45, A103, MP1.1C11), many tumors expressed a more selective antigen

pattern and lacked one or two of these melanoma markers. Interestingly, uveal melanoma can only be partially recognized by the three most widely used immunohistochemical reagents for diagnosis—HMB-45, S-100, and A103 (Melan-A/Mart-1-specific antibody)—as shown by several studies.<sup>10-13</sup> The first two mAbs recognize approximately 25% to 79% of this tumor type.<sup>10</sup> In comparison, our results revealed strong MC1R expression in all tested primary uveal melanoma tissue sections ( $n = 17$ ; Tables 1 and 2) and uveal melanoma cell lines (Fig. 1). Therefore, MC1R may constitute a valuable complementary marker for uveal melanomas to be used in diagnosis and possibly in T-cell-based immunotherapy.

Accumulating evidence indicates that the MC1R ligand  $\alpha$ -MSH, besides being a hormone involved in pigmentation,<sup>17,18</sup> also plays a crucial role in the regulation of immune and inflammatory reactions.<sup>28,29</sup> Indeed, stimulated monocytes and macrophage lines were shown to express MC1R.<sup>15,18,26</sup> Our data indicated that though MC1R is not expressed at detectable levels on fresh monocytes, in vitro stimulation with several cytokines, such as IL-4, GM-CSF, and IL-10, can induce a strong expression of this receptor.<sup>15</sup> In this study, we evaluated whether the surface expression of MC1R in melanoma cell lines may also be modified by proinflammatory cytokines. Treatment of ocular melanoma cells with IFN- $\gamma$  or TNF- $\alpha$  significantly augmented MC1R surface expression. However, the total cellular expression of MC1R was not modified, as demonstrated by flow cytometry analyses on permeabilized cells. In addition, RT-PCR assays on these uveal melanoma cell lines showed no changes in mRNA levels as a result of cytokine treatment (data not shown). It was observed that most melanoma cell lines showed heterogeneous surface expression. In

nonstimulated cultures, two populations, one MC1R negative and one MC1R positive, could be observed. In contrast, melanoma cells treated with stimulating factors such as cytokines or the hormone analogue peptide showed a homogeneous positive expression of MC1R on the cell surface, possibly reflecting an association between MC1R surface expression and cell cycle or activation status of melanoma cells.

Together, the data suggest that the observed increase of MC1R surface expression, in response to cytokine treatment, was caused by a posttranslational regulatory mechanism that seemed not to involve enhanced transcription of the *MC1R* gene. This notion was supported by immunocytochemistry, immunofluorescence, and flow cytometry experiments on OCMS1 cells showing that in the absence of cytokine treatment, MC1R staining locates mainly at the perinuclear area and partially in the cytosol. In contrast, treatment with IFN- $\gamma$  promoted a change in the location of MC1R staining, which now was also found at the cell surface.

The use of mAbs directed to molecules expressed on tumors has been tested in animal models and in clinical trials alone or coupled by drugs and radionuclides.<sup>30</sup> More recently, murine mAbs have been modified by genetic engineering, producing chimeric (ch), humanized (hz), and human mAbs, some of which are used for the treatment of cancer.<sup>31</sup> Several targets have been defined for different tumors, including the receptor of tyrosine-kinase type I and growth factors receptors, such as HER2/neu, which is overexpressed in gastric, ovarian, and pulmonary cancer and in 30% of invasive breast cancer.<sup>32,33</sup> Other strategies include the blocking of the CD20 marker in B lymphomas and the inhibition of angiogenesis-blocking VEGF.<sup>34,35</sup> Finally, the chimeric Ab KM871, directed against gangliosides, recognizes mainly melanoma cells.<sup>36</sup> However, the potential use of antibody-mediated therapy requires target molecule expression on the tumor cell surface. Cytokine-mediated induction of MC1R surface expression on melanoma cells may become clinically relevant. It is known that IFNs can induce the expression of MHC class I and II and of costimulatory molecules on tumor cells and cells from the immune system.<sup>37</sup> In fact, IFN- $\gamma$  (Actimmune; Intermune, Brisbane, CA) has been approved by the Food and Drug Administration (FDA) for the treatment of some immune-related diseases such as chronic granulomatous disease and severe malignant osteopetrosis.<sup>38,39</sup> The high toxicity of IFNs makes it necessary to carefully explore the dose to be used in patients so as to obtain the desired result with the fewest adverse effects. However, the high mortality rate of metastatic disease and the lack of effective treatments justify the exploration of new therapeutic modalities. Results obtained in this work showed that MC1R-specific mAb could be a useful tool in the characterization of ocular malignant melanoma, and its combined use with cytokine treatment for cancer immunotherapy should be considered.

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