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RESEARCH ARTICLE

Induction of metallothionein expression during monocyte to melanoma-associated macrophage differentiation

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Abstract Tumor-associated macrophages (TAMs) play a critical role in melanoma growth and metastasis. Infiltration of TAMs correlates with the poor prognosis of melanoma. TAMs are differentiated from monocytes in response to the tumor microenvironment cue. However, the mechanism how TAMs adapt to the tumor microenvironment after differentiation from monocytes is not fully understood. In addition, specific identification of TAMs in melanoma is difficult because the expression of the most commonly used macrophage marker, CD68, is also expressed in melanoma cells. In an earlier study, we found by gene microarray analysis that seven members of the metallothionein (MTs) family were upregulated in melanoma-conditioned medium induced macrophages (MCIM-Mϕ). MTs have been implicated in zinc metabolism and inflammation. In the present study, we confirmed that expression of metallothionein is induced in M-CSF differentiated macrophages (M-CSF/Mϕ) and MCIM-Mϕ at both the mRNA and protein levels using real-time PCR, immunofluorescence, and western blot analysis. Furthermore, we demonstrated the presence of metallothionein in melanoma tissues *in vivo* and that metallothionein was co-localized with TAMs markers, CD68 and CD163. Finally, we demonstrated the induction of the zinc importer gene Zip8 both in M-CSF/Mϕ and MCIM-Mϕ. Our study identifies metallothionein as a novel marker for TAMs and suggests that metallothionein might play important roles in macrophage adaptation and function in the tumor microenvironment.

Keywords melanoma, macrophages, metallothionein

Introduction

Compelling evidence suggests that tumor-associated macrophages (TAMs) within the tumor microenvironment play essential roles in tumor progression and metastasis (Pollard, 2004; Lewis and Pollard, 2006; Sica et al., 2007; Mantovani and Sica, 2010; Qian and Pollard, 2010). The increased infiltration of TAMs in melanoma has been associated with poor prognosis (Bröcker et al., 1988; Bernengo et al., 2000; Mäkitie et al., 2001; Varney et al., 2005), and expression of TAMs markers, CD68 and CD163 in melanoma tissue and serum predicts a poor prognosis for early stage melanoma (Jensen et al., 2009). TAMs produce a large variety of cytokines and chemokines, proteolytic enzymes, extracellular matrix molecules and growth factors that have profound effects on tumor cells and other components in the tumor microenvironment. In melanoma, TAMs have been reported to play important roles in every stage of melanoma development, including melanoma initiation, anti-tumor immunity, angiogenesis and metastasis (Bernengo et al.,

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2000; Varney et al., 2005; Gazzaniga et al., 2007; Zaidi et al., 2011; Wang et al., 2012).

Melanoma cells produce chemokines and cytokines that recruit circulating blood monocytes to tumor sites. The differentiation of monocytes into TAMs is dependent on factors secreted by melanoma cells, such as macrophage colony-stimulating factor (M-CSF), chemokine (C-C motif) ligand 2, leukemia inhibitory factor and IL-6 (Niida et al., 1999; Duluc et al., 2007; Roca et al., 2009; Solinas et al., 2010). There are numerous changes that occur during the differentiation from monocytes to TAMs. For instance, in addition to producing factors that have a profound effect on tumor growth, angiogenesis and metastasis, macrophages have been shown to produce anti-apoptotic factors that enable them adapt to the tumor microenvironment and enhance their survival. One such group of factors is the metallothioneins (MTs). MTs belong to a family of small, cysteine-rich proteins that assist in cellular homeostasis and protect against injury caused by oxidative stress. MTs expression is induced by heavy metals and inflammatory factors. Once produced, these proteins function as free radical scavengers, reservoirs for essential heavy metals such as zinc, and play a key role in immunomodulation. The expression of several MTs is downregulated in many types of tumor cells, including melanoma, due to their DNA methylation (Henrique et al., 2005; Koga et al., 2009). MTs have also been implicated to play an important role in the regulation of macrophage function. For example, it has been shown that macrophages from metallothionein null mice have defects in phagocytosis and cytokine production (Sugiura et al., 2004). However, whether MTs play a role in modulating TAMs is not known.

Studying the effect of TAMs on melanoma growth and metastasis is limited by a lack of definitive biomarkers to identify TAMs. The most commonly used TAMs marker, CD68, is also expressed in melanoma cells. It becomes necessary, therefore, to identify molecules that are exclusively expressed in TAMs and not in tumor cells. Genes that have been silenced by epigenetic modification in tumor cells but that remain expressed in TAMs will be the ideal candidates for biomarkers.

In the present study, we confirmed the induction in TAMs of MTs by microarray gene profiling signature in TAMs, and show that MTs are induced in melanoma-conditioned medium induced macrophages (MCIM-Mϕ). We verified this gene expression pattern in TAMs by real-time PCR. Furthermore, we found increased expression of metallothionein in inflammatory cells and show that metallothionein is localized to the cytoplasmic compartment. Finally, we found that metallothionein is partially co-localized with CD68, and near fully co-localized with a more specific marker, CD163. In summary, our study indicates a potentially important role for metallothionein in maintaining TAMs homeostasis and identifies metallothionein as a potential novel marker for TAMs.

Materials and methods

Differentiation of macrophages

In conformance with institutional policies regarding human experiments, monocytes were obtained from healthy volunteers by leukapheresis followed by countercurrent elutriation (AIDS Research Human Immunology Core at the University of Pennsylvania, USA). To make M-CSF induced macrophages, monocytes were incubated in the presence of RPMI1640 medium supplemented with 100 U/mL penicillin/streptomycin, 10 mm HEPES, 10% heat-inactivated fetal bovine serum (FBS), 5×10^{-5} mM 2-mercaptoethanol (2-ME) with M-CSF (10 ng/mL, R&D systems) for 7 days as described (Wang et al., 2012). Half of the medium was replaced with fresh medium at day 3 as described.

To make melanoma-conditioned medium induced macrophages, we first made modified-conditioned medium with concentrated melanoma medium from 1205Lu melanoma cells (40 : 1) and then diluted in complete RPMI medium (1 : 80). Monocytes were then incubated in the presence of modified-conditioned medium for 7 days. Half of the medium was replaced with fresh medium at day 3 as detailed in reference (Wang et al., 2012).

Real-time PCR

Total RNAs were isolated using the RNeasy Mini kit (Qiagen). cDNA was prepared using oligo (dT) primers and Superscript reverse transcriptase (Invitrogen). Real-time PCR was conducted on a 7900 Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). All real-time PCR was performed using 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and then finally, extension for 10 min at 72°C. Primers used for real-time PCR were same as referenced (Raymond et al., 2010).

Flow cytometry analysis

Expression of MT1G in monocytes and MCIM macrophages was measured by intracellular flow cytometry analysis. Cells were incubated in RPMI medium containing GolgiStop (1 μg/mL) for 2 h. Cells were fixed/permeabilized, and stained with rabbit anti-human metallothionein polyclonal antibody and isotype control, and followed by FITC-conjugated anti-rabbit IgG. Cells were analyzed using a FACScalibur$^{\text{TM}}$ (Becton Dickinson) and analyzed by Flowjo software.

Immunohistochemistry

Formalin-fixed, paraffin-embedded human melanoma tissues were from the University of Pennsylvania under an approved

IRB protocol. For metallothionein staining on primary melanoma lesions, tissues were deparaffinized through a series of xylene and graded alcohols, boiled in 10 mM citrate buffer (pH 6.0) for 10 min at 92°C–95°C and equilibrated in PBS for antigen retrieval. Subsequently, the slides were incubated with the rabbit anti-human metallothionein polyclonal antibody (1:200, Santa Cruz Biotechnology) overnight at 4°C in a humidified chamber, followed with VECTASTAIN Elite ABC Kit and Vector AEC substrate (Vector Laboratories).

Immunofluorescence

For staining metallothionein in monocytes and macrophages, cells were cytocentrifuged (Cytospin 2 Shandon, Frankfurt, Germany) onto microscope slides for 5 min at 500 rpm. Cells were then fixed with 4% formaldehyde, and incubated with anti-human metallothionein antibody followed by incubation with Alexa Flour 488 secondary antibodies (Molecular Probe). Vectashield mounting media containing DAPI nuclear stain (Vector Laboratories) was used to mount the slides with coverslips.

For immunofluorescence co-staining for expression of metallothionein with CD68 and CD163, primary melanoma tissues were treated the same as described in immunohistochemistry. After incubation with the anti-metallothionein antibody (1:200, Santa Cruz Biotechnology) at 4°C, sections were incubated with biotin conjugated goat anti-rabbit IgG and Alex 488-conjugated streptavidin for 30 min at room temperature in a humidified chamber. The slides were subsequently incubated with the mouse anti-CD68 (DAKO) or mouse anti-CD163 (Abcam) antibodies diluted in 0.5% BSA PBS for 2 h at room temperature, followed by Alex 568-conjugated anti-mouse IgG mAb. Slides were counterstained with DAPI and covered with polyvinyl-alcohol mounting medium (DABCO).

Zinc measurements

Intracellular zinc levels were determined in monocytes, M-CSF/Mф and MCIM-Mф with Newport Green DCF diacetate, a cell-permeable, fluorescent, zinc specific probe (Invitrogen/Molecular Probes, Eugene, OR, USA). 1×10^6 cells were incubated for 45 min with Newport Green (10 μM) at room temperature. Cells were then washed twice and re-suspended in FACS wash buffer, and analyzed by flow cytometry.

Results

Induction of metallothionein family genes during monocyte differentiation from monocytes to MCIM-Mф

To investigate the gene profile changes that occur during monocyte to melanoma-associated differentiated macrophages, we first used an *in vitro* system to differentiate monocytes to macrophages using melanoma-conditioned medium (MCM), which we designated as MCMI-Mф. MCMI-Mф showed a mixture of M1 and M2 macrophage phenotypes, and displayed many functional characteristics of TAMs, including inhibition of T cell proliferation and the promotion of melanoma cell invasion (Wang et al., 2012). Gene expression microarray analysis on MCMI-Mф revealed that seven members of the metallothionein family, including MT1G, MT1E, MT1H, MT1A, MT2A, MT1F and MTE, were among the top ranked 100 upregulated genes in MCMI-Mф compared to monocytes. In addition, two other MT gene family members, MT1E and MT1, were also upregulated in TAMs compared to monocytes (Fig. 1A). We performed a real-time PCR assay to validate the expression of genes identified by microarray analysis. Consistent with the microarray data, we found significantly increased expression of MT1G, MT1E, MT1H, MT1A, MT2A in MCMI-Mф compared to monocytes. Interestingly, M-CSF/Mф also expressed higher levels of M1TG, MT1E, MT1H, MT1A, and MT2A, but to a lesser amount than MCMI-Mф (Fig. 1B). Our data demonstrate that mRNA expression of MTs genes was induced during differentiation of monocytes to macrophage.

Increased protein expression of metallothionein in MCIM-Mф compared to monocytes

Next, we analyzed by flow cytometry the expression of metallothionein in TAMs using a specific polyclonal anti-human metallothionein antibody. This antibody recognizes multiple mammalian metallothionein proteins. Consistent with real-time PCR results, intracellular staining of metallothionein indicated that MCMI-Mф express a higher level of metallothionein, while monocytes express undetectable amounts of metallothionein (Fig. 2A). It has been reported that the cellular localization of metallothionein protein is related to its function. For example, cytoplasmic metallothionein has an anti-apoptosis role, while nuclear metallothionein expression plays a role in pro-apoptosis. To identify the subcellular localization of metallothionein expression in differentiated macrophages, and to further confirm expression of metallothionein at the protein level, we performed immunofluorescence staining with an anti-metallothionein antibody. Metallothionein labeling was detected in the cytoplasmic compartment of TAMs, further indicating that metallothionein protein might play a role in protecting TAMs from pro-apoptotic stimulation within the tumor microenvironment (Fig. 2B). Finally, we investigated the expression of metallothionein in primary melanoma lesions in vivo. It has been reported that metallothionein is absent in many types of cancer cells, including melanoma, because of the hypermethylation of this gene in tumor cells. As expected, in our

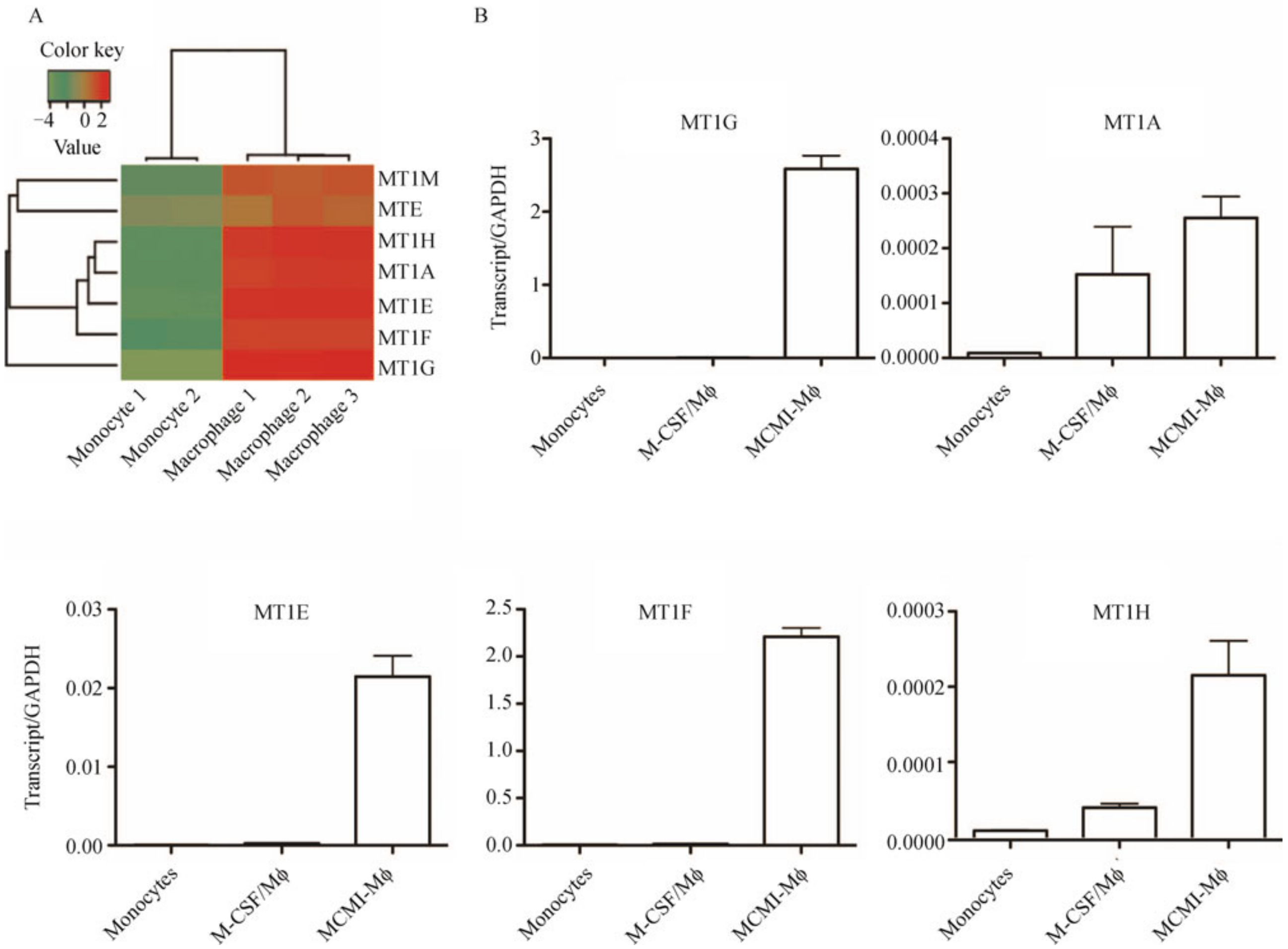

Figure 1 Induction of expression of metallothionein genes in M-CSF/Mϕ and MCIM-Mϕ. (A) Heatmap of gene expression of metallothionein genes in MCIM-Mϕ compared to monocytes. (B) Monocytes from healthy donors were incubated with melanoma-conditioned media for 7 days. Cells were harvested, total RNA isolated and real-time PCR was used to verify expression of MT genes in M-CSF/Mϕ and MCIM-Mϕ, including MT1G, MT1A, MT1E, MT1F and MT1H. Data are representative of 3 independent experiments with 3 healthy donors.

experiments, immunohistochemistry staining indicated that metallothionein is expressed in the inflammatory cells, but not expressed in tumor cells (Fig. 2C).

Metallothionein co-localizes with TAMs marker, CD68 and CD163

To confirm that metallothionein expression is indeed associated with TAMs in human melanoma tissues, we performed double staining of metallothionein with the most commonly used TAMs markers, CD68 and CD163 on primary human melanoma tissues. Our results indicate that metallothionein was expressed in most CD68 positive cells (Fig. 3A); however, we did not observe a complete overlap between metallothionein and CD68 expressing cells, presumably because some melanoma cells were CD68 positive as reported. Metallothionein was expressed in most CD163 positive cells (Fig. 3B). In addition, similar to what we observed with the cellular staining (Fig. 2B), we detected that metallothionein is localized in the cytoplasmic compartment. These data suggested that metallothionein is a novel marker for TAMs.

Increased expression of intracellular zinc content and zinc transporter gene Zip8 in macrophages compared to monocytes

One of the major activities of metallothionein is the maintenance of zinc homeostasis, due to its ability to modulate intracellular zinc levels. Based on the increased metallothionein gene expression in MCMI-Mϕ, we investigated whether metallothionein expression would impact zinc retention and homeostasis in monocytes and TAMs. Using the cell-permeable zinc probe DCF, we found a significantly increased level of intracellular zinc in MCMI-Mϕ compared to monocytes, indicating greater zinc levels in TAMs (Fig. 4A). Next, we tested whether a zinc transporter gene Zip8 was also increased in MCMI-Mϕ. Real-time PCR analysis

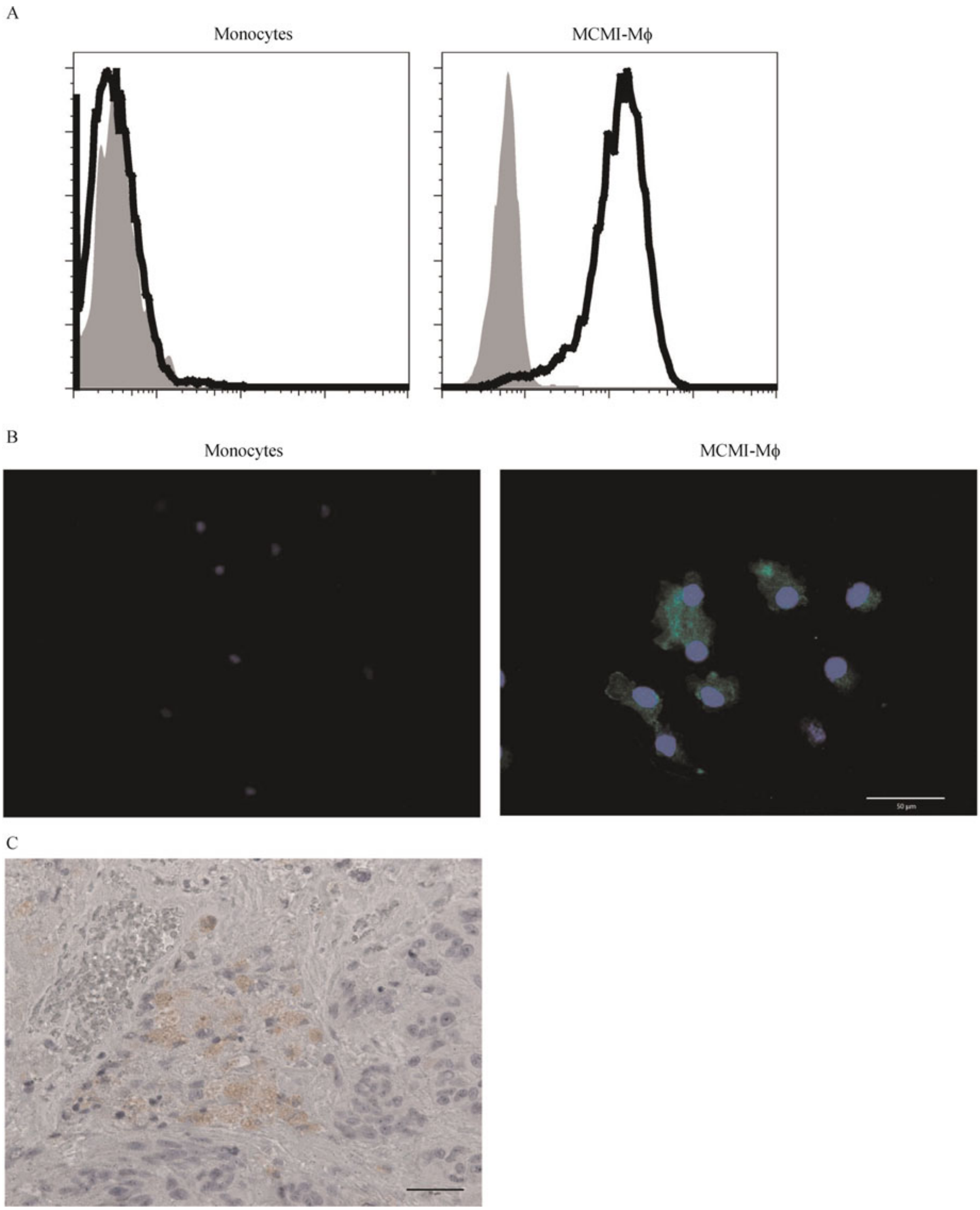

Figure 2 Expression of metallothionein in MCIM-Mϕ. (A) Expression of metallothionein in MCIM-Mϕ. FACS analysis was performed to quantify intracellular metallothionein expression in monocytes and MCIM-Mϕ. Gray shadow fills = isotype matched control; black lines = primary antibodies. (B) Monocytes and MCIM-Mϕ were fixed, cytospinned on glass slides, and stained with rabbit anti-metallothionein polyclonal antibody for immunofluorescence microscopy. Scale bar = 50 μm. (C) Formalin-fixed, paraffin embedded melanoma tissue sections were deparaffinized followed by antigen retrieval in citrate buffer. Melanoma tissues were then stained with anti-metallothionein antibody, and revealed that metallothionein is expressed in inflammatory cells, but not in tumor cells of primary melanoma lesion. Scale bar = 50 μm. Data are representative of 4 primary melanoma lesions.

showed that the expression of Zip8 gene is higher in MCMI-Mϕ than monocytes. MCSF/Mϕ also express high level of Zip8, but less than MCMI-Mϕ (Fig. 4B). Our data suggest that TAMs sustain high levels of intracellular zinc. Taken

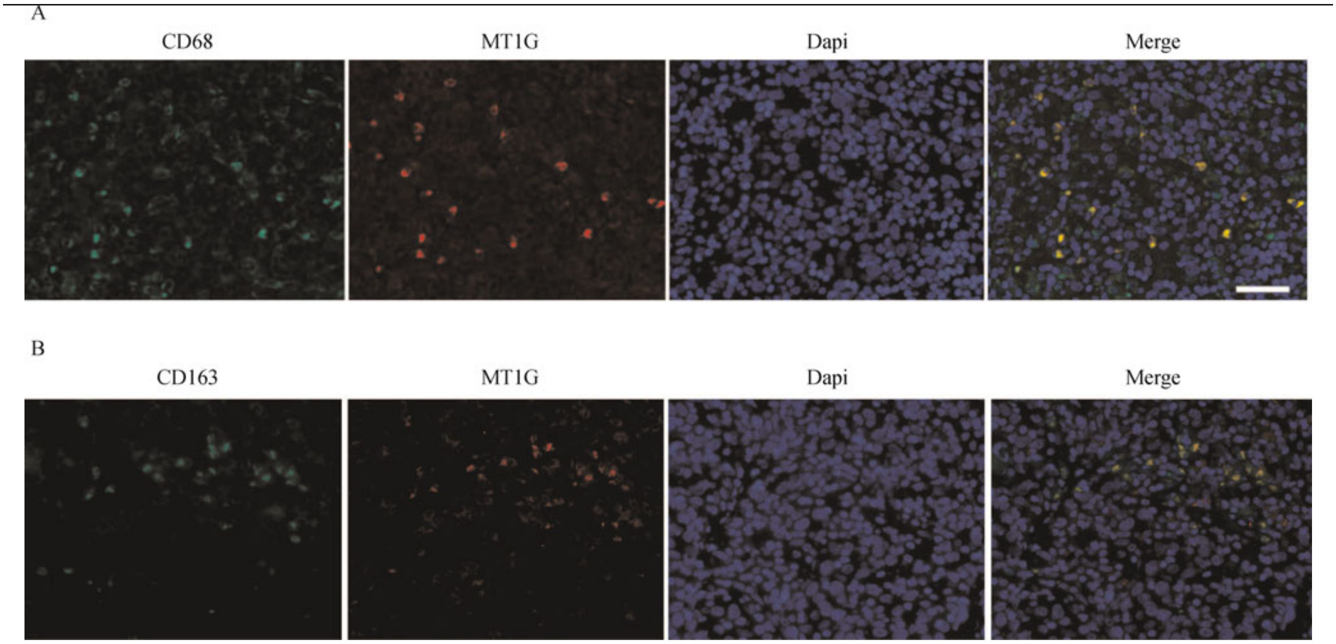

Figure 3 Metallothionein is co-localized with CD68 and CD163 in melanoma tissues. Co-staining of CD68 (A) and CD163 (B) with metallothionein in human melanoma patient tissues. Melanoma tissues were treated as in Figure 3C. Anti- metallothionein and anti-CD68, CD163 antibodies were used to measure the co-localization of metallothionein with CD68 and CD163. Data are representative of 3 primary melanoma lesions.

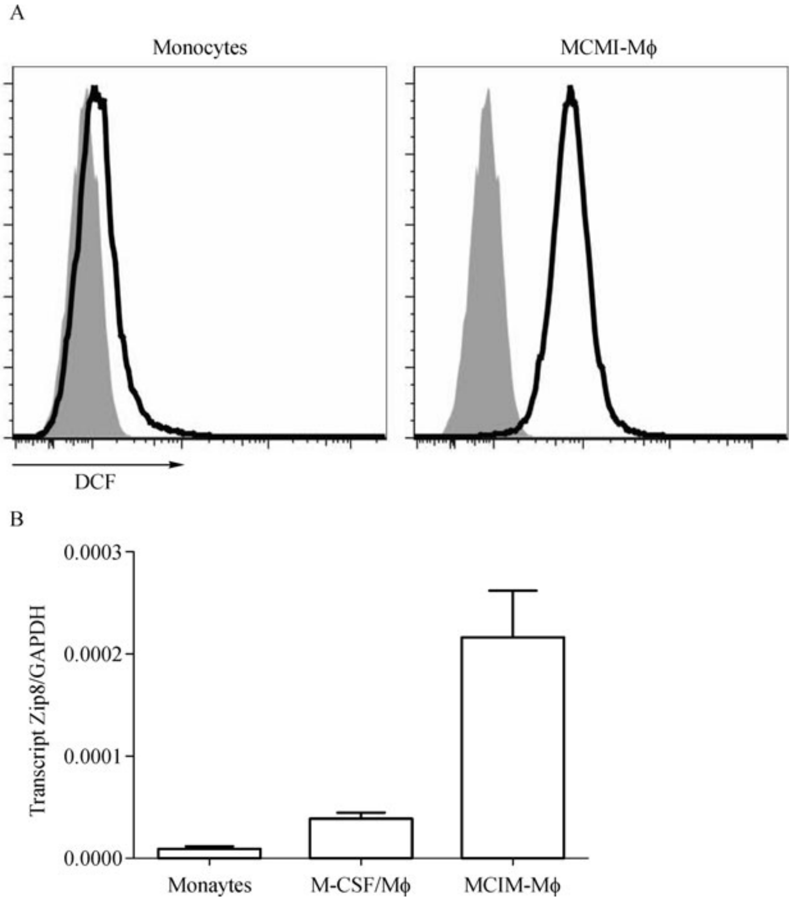

Figure 4 Increased expression of intracellular zinc in MCIM-Mϕ. (A) DCF uptake assay was used to analyze the level of intracellular zinc in monocytes and MCIM-Mϕ. Gray = unstained control; black lines = DCF staining. (B) Real-time PCR was used to analyze the expression of zinc importer gene Zip8 in monocytes, M-CSF/Mϕ and MCIM-Mϕ.

together, the data support a direct link between TAMs differentiation, elevated MT gene expression, and increased zinc levels in TAMs.

Discussion

In the present study, we demonstrated an elevation in the expression of several members of metallothionein family during monocyte to MCIM-Mф differentiation. Expression of metallothionein is regulated by many factors produced by tumor cells or tumor stroma cells, such as VEGF, IL-6, and TNF-alpha (De et al., 1990; Joshi et al., 2005). In addition, metallothionein is an oxygen-regulated protein, and, therefore, induced in the hypoxic tumor microenvironment (Raleigh et al., 1998; Murphy et al., 1999; Raleigh et al., 2000; Yamasaki et al., 2007a). The induction of metallothionein in MCIM-Mф might be due to both melanoma cell produced factors and hypoxic melanoma environment. There are conflicting reports about the expression of metallothionein in melanomas. Several reports from Zelger's group demonstrated that expression of metallothionein is correlated with tumor thickness and invasiveness and is a poor prognostic factor in melanomas (Weinlich et al., 2003; Weinlich et al., 2006; Weinlich and Zelger, 2007; Weinlich et al., 2007; Weinlich, 2009). These studies, however, did not determine whether the metallothionein is expressed in tumor cells or other types of cells. In our study, we found that metallothionein is not expressed in melanoma cells, but in TAMs in melanoma tissues (Fig. 3). The discrepancy between our results and those from the aforementioned studies might be due to the difference of antibody specificity. Our data are consistent with several recent studies that have demonstrated that metallothionein is downregulated in melanoma due to promoter methylation that inactivates the expression of metallothionein (Henrique et al., 2005; Koga et al., 2009). Furthermore, it has been reported that expression of some members of the metallothionein family is downregulated in many types of cancers, and the status of metallothionein is associated with prognostic status (Ghoshal et al., 2002; Deng et al., 2003; Huang et al., 2003; Henrique et al., 2005; Tse et al., 2009).

The major function of metallothionein is to maintain metal homeostasis, which is necessary to optimal cellular and organismal survival. We can presume that expression of metallothionein in TAMs may play a role to maintain zinc homeostasis and promote adaption to the tumor microenvironment. In addition, metallothionein is a stress response protein that plays a crucial role in protection against oxidative damage, heavy metal toxicity, and other stress responses. The location of metallothionein in the cells correlates with an anti- or pro- apoptotic outcome. Wallace et al. reported that the presence of metallothionein in the cytoplasmic compartment is important to protect against DNA damage and stress induced apoptosis (Levadoux-Martin et al., 2001). Because our data demonstrated that metallothionein is localized in the cytoplasm in MCMI-Mф (Fig. 2B) and melanoma tissues (Fig. 3), we conclude that metallothionein may also contribute to the protection of TAMs from apoptosis. We were not able to compare the overall sensitivity of macrophages and monocytes to heavy metal induced apoptosis because the baseline for sensitivity for induction of apoptosis between the two cell types is so different, with the sensitivity of monocytes being significantly higher than that of macrophages.

Intracellular zinc level has been proposed as a novel intracellular second messenger and plays important roles in cell proliferation, differentiation, survival and apoptosis (Yamasaki et al., 2007b; Colvin et al., 2010). In addition, it has been reported that zinc level is induced in early stage of macrophage differentiation and may play a role in macrophage differentiation (Glesne et al., 2006). Deficient of level of zinc also inhibit many function of macrophages, including intracellular killing, cytokine production, and phagocytosis (Shankar and Prasad, 1998). Our data indicated that intracellular zinc level is upregulated in MCMI-Mф (Fig. 4A). Additionally, we found that expression of the zinc importer gene, Zip8, is induced in MCIM-Mф compared to monocytes. These data suggested that zinc might also play a role in MCIM-Mф and maintain the functions of MCIM-Mф.

Two most commonly used biomarkers for macrophages are CD163 and CD68. CD163 is a protein of the scavenger receptor cysteine-rich (SRCR) superfamily whose exclusive expression is limited to monocytes and macrophages in humans. The CD68 protein is one member of the lysosomal/endosomal-associated membrane glycoprotein (LAMP) family with high expression in human monocytes and macrophages. Although CD163 seems thus far to have remained a faithful biomarker that can delineate between macrophages and other cell types, CD68 however has come into some controversy, specifically in terms of its expression in melanoma. Leader and colleagues suggested that CD68 is not an effective marker in specific settings; they found that it was expressed in 5 out of 7 malignant melanoma samples (Cassidy et al., 1994). Likewise, Wheler and colleagues found reactivity of CD68 in 38 of 42 primary, 5 of 6 desmoplastic, and 11 of 12 metastatic melanomas (Shah et al., 1997). In a study done by Adsay et al., they found that in samples of primary and metastatic melanoma, the positive immunoreactivity of CD68 was almost as diffuse as traditional melanoma markers (S100, HMB45, and NKI-C3), though weaker in intensity (Pernick et al., 1999). With the questionable nature of the cross expression of CD68 in melanoma and macrophages, a more exclusive biomarker must be identified and used. Our result further indicated that CD68 is not a specific marker for TAMs (Fig. 3), and indicated that metallothionein is exclusively expressed in TAMs (Fig. 3B) and can be used as a biomarker for TAMs.

In conclusion, our results indicated that metallothionein is induced during differentiation from monocyte to macrophage, that metallothionein may play a role in modulation of metal homeostasis and stress induced apoptosis, and finally, that metallothionein can be used a novel marker for TAMs.

Authorship

Contribution: T.W., Y.G., designed, performed research and drafted the manuscript; R.Z., X.M., A.L-C., B.G., Z.G., Z.W., performed experiments; X.X. provided melanoma tissues. M.H., L.J.M., helped organize the manuscript. R.E.K. designed research and drafted the manuscript.

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