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Original Contribution

Maintenance of immune hyporesponsiveness to melanosomal proteins by DHICA-mediated antioxidation: Possible implications for autoimmune vitiligo

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ABSTRACT

Melanocyte destruction in the skin of vitiligo patients has been considered to be a consequence of an autoimmune response against melanosomal proteins. However, little is known about the molecular mechanisms by which the immune system recognizes these sequestered intracellular self-proteins, which are confined in specialized organelles termed melanosomes, and is provoked into an autoimmune response to melanocytes. Here, we utilize a sucrose density-gradient ultracentrifugation protocol to enrich melanosomal components from dopachrome tautomerase (Dct)-mutant or wild-type melanocytes exposed to a pulse of hydrogen peroxide at a noncytotoxic concentration to evaluate their immunogenicity in mice challenged with the corresponding melanosomal proteins. The results demonstrate that enhanced humoral and cellular immune responses to a challenge with late-stage melanosomal proteins, especially with those derived from Dct-mutant melanocytes, are found in the immunized mice. To elucidate whether a reduced 5,6-dihydroxyindole-2-carboxylic acid (DHICA) content in melanin might cause a loss in antioxidative protection to the proteins, we incubated these melanosomal proteins in vitro with synthetic 5,6-dihydroindole (DHI)–melanin or DHI/DHICA (1:1)–melanin and then used them to immunize mice. T cell proliferation and IgG antibody responsiveness to the challenges were significantly induced by melanosomal proteins treated with DHI–melanin, but not by those treated with DHI/DHICA (1:1)–melanin. Moreover, we observed that melanosomal proteins derived from Dct-mutant melanocytes are subject to oxidative modifications that alter their antigenic configurations to attain an enhanced immunogenicity compared with those derived from wild-type melanocytes. From these results, we conclude that DHICA-mediated antioxidation plays a critical role in the maintenance of immune hyporesponsiveness to melanosomal proteins.

Functional melanocytes disappear from the skin of vitiligo patients by a mechanism that is not fully understood [1–4]. It is generally accepted that the loss of histochemically recognizable melanocytes in the involved skin is the result of their destruction, caused at least in part by altered humoral and/or cellular immune reactions against the melanocytes per se [2]. Skin-homing autoreactive T cells [3] and autoantibodies [4] to melanocytes have been detected in the lesions and/or the sera of vitiligo patients, directed against various melanocyte antigens such as tyrosinase, tyrosinase-related protein 1 (Tyrp1), and dopachrome tautomerase (Dct/Tyrp2). However, it deserves further scrutiny to characterize how immunoglobulins recognize intracellular self-proteins to mediate cellular destruction and how these sequestered organelle antigens, which are confined in specialized compartments termed melanosomes, are exposed to the immune system at the onset of the disease [5]. The emerging view is that vitiligo melanocytes may have an intrinsic defect that makes them more susceptible to reactive oxygen species (ROS) and to overproduction of hydrogen peroxide (H_2O_2) found in the vitiligo epidermal milieu, which possibly induces the intracellular melanosomal antigens to be released subsequent to oxidative damage of melanocytes [6–8]. Despite the fact that exposure of melanosomal proteins to antigen-presenting cells might occur, an immune response to these normal self-antigens would presumably be difficult to provoke as a consequence of immune tolerance. It is possible that the occurrence of protein oxidative modification in vitiligo melanocytes is indispensable for the efficient induction of those immune responses [9].

A recent study demonstrated that inactivation of Dct lessens the radical-scavenging potential in Dct-deficient melanocytes and increases their vulnerability to oxidative damage [10]. Dct is a critical enzyme in the melanogenesis pathway that isomerizes the intermediate dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and influences the proportion of the DHICA monomer incorporated

Abbreviations: Dct, dopachrome tautomerase; Tyr, tyrosinase; Tyrp1, tyrosinase-related protein 1; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DHI, 5,6-dihydroindole; ROS, reactive oxygen species; H_2DCFDA, 2′,7′-dichlorofluorescin diacetate.

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into melanin with the 5,6-dihydroxyindole (DHI) polymer, thereby generating a DHICA-rich eumelanin that confers a potent hydroxyl radical scavenging activity [10]. The spontaneous *slaty* mutation in the mouse Dct gene dramatically decreases the catalytic activity of the mutant enzyme compared with wild-type Dct [11,12]. In this study, we attempted to define the sensitivity of Dct-mutant melanocytes to oxidative stress, the immunogenicity of melanosomal proteins derived from Dct-mutant melanocytes after treatment with H_2O_2, as well as the protection from oxidative insults by synthetic DHICA-rich melanin. Our results show that oxidative stress significantly enhances the immunogenicity of melanosomal proteins derived from Dct-mutant melanocytes and that DHICA-mediated antioxidation plays a role in the maintenance of immune hyporesponsiveness to melanosomal proteins.

Materials and methods

Cell lines and cell culture

Murine melan-a melanocytes were a kind gift from Professor Dorothy C. Bennett (St. George's Hospital, London, UK) [13]. They were originally derived from C57BL/6J (black, a/a) mice and are routinely passaged in complete RPMI 1640 medium with 5% heat-inactivated fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μM mercaptoethanol, 2 mM L-glutamine, and 200 nM phorbol myristate acetate (PMA). Murine Dct-mutant (Dct^{slt}/Dct^{slt}) melanocytes were generously provided by Dr. Vincent J. Hearing, (Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health (NIH), Bethesda, MD, USA) [11]. They were originally derived from dorsal skins of 1-day-old mice carrying the *slaty* mutation in the Dct gene. Dct-mutant melanocytes are cultured in the same medium used for melan-a melanocytes, as previously described [14].

H_2O_2-pulse treatment and intercellular ROS assay

Both types of melanocytes (2×10^5) were cultured in six-well plates in complete RPMI 1640 medium. For the H_2O_2-pulse treatment [15], the medium was changed to D-PBS with or without 100 μM H_2O_2 (Sigma–Aldrich, St. Louis, MO, USA) for 1 h. Intercellular ROS levels were measured using the oxidation-sensitive fluorescent dye 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) as described previously [10]. Briefly, equal numbers of cells were loaded with 10 μM H_2DCFDA (Sigma) for 20 min at 37 °C after exogenous H_2O_2-pulse treatment and then were washed with D-PBS twice. It should be noted that the nonfluorescent ester H_2DCFDA penetrates into cells and undergoes deacetylation to DCFH by cellular esterases. The DCFH probe is rapidly oxidized to the highly fluorescent compound 2′,7′-dichlorofluorescin (DCF) by ROS. ROS levels are expressed as the fluorescence intensity of DCF measured at an excitation of 495 nm and an emission of 527 nm using a Hitachi F4500 spectrofluorimeter (Hitachi Ltd., Tokyo, Japan).

Fontana–Masson staining

Fontana–Masson staining was performed to assess the amount of melanin in Dct-mutant *slaty* melanocytes and in melan-a melanocytes, as described previously [10,16]. Monolayer melanocytes grown on coverslips were fixed with cold acetone for 10 min and then were stained with a 10% silver nitrate solution for 2 h in a dark chamber. After being rinsed in water, the coverslips were placed in 0.1% gold chloride solution for 5 min, rinsed in water, and then placed in 5% sodium thiosulfate for an additional 5 min. After being rinsed with water, the coverslips were counterstained with eosin red.

Purification of melanosomes by sucrose density-gradient ultracentrifugation

Preparation of cellular homogenates and purification of melanosomes were performed according to the method of Kushimoto et al. [17]. Briefly, confluent monolayers of Dct-mutant melanocytes or melan-a melanocytes were treated with D-PBS containing 100 μM H_2O_2 for 1 h. After the pulse treatment with H_2O_2, the cells were harvested with 0.25% trypsin/0.02% EDTA and washed once in 0.25 M sucrose by centrifugation at 1000*g* for 5 min at 4 °C. They were then homogenized on ice using 20 strokes of a Dounce glass–glass homogenizer and were centrifuged at 1000*g* for 10 min at 4 °C. The cellular homogenate was then layered on a discontinuous gradient of 1.0, 1.2, 1.4, 1.5, 1.6, 1.8, and 2.0 M sucrose (in 10 mM Hepes, pH 7.4) and centrifuged at 100,000*g* in a Beckman SW28 swinging-bucket rotor for 1 h at 4 °C. Early and late melanosomes were recovered from the 1.2–1.4 M and the 1.6–1.8 M sucrose interfaces, respectively. Fractions were collected and analyzed for enzyme activity, ultrastructure, melanogenic protein expression, and immunogenicity in vivo, as detailed below.

Transmission electron microscopy

Dct-mutant *slaty* melanocytes and melan-a melanocytes in the exponential growth phase were harvested and fixed with 2.5% (v/v) glutaraldehyde for 24 h at 4 °C, collected by centrifugation, and then washed twice with cold PBS. All samples were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for 1 h, dehydrated through a graded ethanol series, and embedded in Epon at 60 °C for 48 h. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and then examined in a transmission electron microscope (Tecnai G2, FEI Co., Eindhoven, The Netherlands) [18].

Western blot analysis

For Western blotting, the cells were washed in PBS and lysed in extraction buffer containing 1% Nonidet P-40, 0.01% SDS, and a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Protein contents were determined with a BCA assay kit (Pierce, Rockford, IL, USA). Equal amounts of each protein extract (10 μg per lane) were resolved using 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). After transblotting onto Immobilon-P membranes (Millipore, Bedford, MA, USA) and blocking in 5% nonfat milk in saline buffer, the membranes were incubated with αPEP7, αPEP1, or αPEP8 (gifts from Dr. Vincent J. Hearing at the NIH), each at a 1:2000 dilution except for αPEP7 (1:1000), or with an anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-VADC1/porin antibody (Abcam, Cambridge, MA, USA) (used as loading controls for whole-cell lysates or subcellular mitochondrial components) for 1 h at room temperature. The membranes were then washed and incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (Pierce) at a dilution of 1:2000 for 1 h at room temperature. The membranes were then washed and specific bands were visualized by a chemiluminescent reaction (ECL; Amersham, Piscataway, NJ, USA) [19].

Enzyme activity analyses of tyrosinase, Dct, and catalase

Tyrosinase activity was assayed as dopa oxidase activity using a modification of the method described by Ando et al. [20]. Approximately 10^7 cells were pelleted and lysed with extraction buffer, and 100 μl of each cell lysate was pipetted into a 96-well plate on ice in triplicate. One hundred microliters of 0.1 M phosphate buffer (pH 6.8) containing 0.1% 3,4-dihydroxy-L-phenylalanine (L-dopa; Sigma) was then added into each well and mixed with the lysates at 37 °C in the dark for 5 min. The plates were read at 475 nm using a microplate reader (Wallac1420; PerkinElmer, Waltham, MA, USA) to estimate the amount of dopachrome generated from dopa. Corrections for the

autoxidation of L-dopa in controls were made. Specific activity is defined as the amount of dopachrome formed (absorbance at 475 nm) per minute and is expressed as a percentage of control.

The spectrophotometric assay of Dct activity was carried out using dopachrome as the substrate, as described previously [21,22]. Dopachrome (0.5 mM) was prepared by mixing 100 μl ice-cold 1 mM L-dopa in sodium phosphate buffer (10 mM, pH 6.8) containing 0.1 mM EDTA and 0.1 mM phenylthiourea (Sigma) with 100 μl 2 mM sodium periodate (Sigma) immediately before use. Fifty microliters of dopachrome solution was added to 50 μl cell lysate in 96-well plates and was incubated for 8 min at 37 °C. The plates were read at 475 nm using a microplate reader (Wallac1420; PerkinElmer) to monitor the disappearance of L-dopachrome. Blanks were performed without enzyme to estimate the rate of the spontaneous absorbance decrease at A_{475}. Dct activity is defined as the amount of dopachrome decrease (absorbance at 475 nm) per minute and is expressed as a percentage of control.

Catalase activity was determined spectrophotometrically using a commercial catalase analysis kit (Beyotime Biotechnology Co., Nanjing, China), as described previously [23]. Briefly, cell extract samples were treated with excess hydrogen peroxide for decomposition by catalase for a specific time, after which the remaining H_2O_2 coupled with a substrate was treated with peroxidase to generate a red product, *N*-4-antipyryl-3-chloro-5-sulfonate-*p*-benzoquinone monoimine, which absorbed maximally at 520 nm. The H_2O_2 consumption per minute in the buffer was converted to units of enzymatic activity on the basis of a standard curve obtained testing scalar units of bovine catalase. Units were corrected for protein content of cell extract.

Immunologic challenge, determinations of T cell proliferation, and antibody titer

Balb/c × C57BL/6 (CB6, $H^{2d\times2b}$) F1 mice were purchased from Beijing Weitong Lihua Experimental Animal Technology Co. (Beijing, China) at 6–8 weeks of age and were housed in pathogen-free microisolator cages in our animal facility. All animal procedures used in this study conformed to NIH guidelines and were approved by the ethics committee of Wuhan University.

Early or late melanosome fractions were further sonicated to achieve native melanosomal proteins that were used for subsequent immune challenge in mice. For antioxidative protection of the melanin enriched with carboxylic acid groups on melanosomal proteins, 200 μg of synthetic DHI–melanin or DHI/DHICA (1:1)–melanin (also referred to as DHICA-rich melanin) [24] was bound to 50 μg of melanosomal proteins after 30 min of incubation on ice and was then pulsed with 100 μM H_2O_2 for 1 h at 37 °C. Native/oxidized melanosomal proteins (50 μg) were emulsified with an equal volume of complete Freund's adjuvant (Sigma) and were subcutaneously injected into one hind footpad and the base of the tail of each mouse. Two weeks later, the mice were boosted intraperitoneally with 25 μg of the corresponding antigen in PBS. Antibody responses were measured from sera collected 1 week after boosting. We also used chicken ovalbumin, a well-defined model antigen containing an immunodominant epitope in H-2b mice, as a positive control in each assay [25–27].

Serum melanosomal protein-specific IgG responses were determined by ELISA, as previously described [25]. Five micrograms of purified protein corresponding to challenged mice was coated onto immuno 96-microwell plates (Nunc, Apogent, Rochester, NY, USA). The titers of antimelanosomal protein antibodies were calculated as the dilution of the test serum to the end point.

T cell proliferation analysis was carried out by standard methods as previously described [25,26]. In brief, T cells isolated from the draining popliteal lymph nodes of each mouse were cultured in triplicate at 1×10^5/well in 96-well plates with multiple doses of melanosomal proteins in RPMI 1640 (Gibco, Grand Island, NY, USA) containing 2% fetal bovine serum. After 48 h at 37 °C, [^{3}H]thymidine (Shanghai Institute of Atomic Energy, Shanghai, China) was added and the cultures were harvested 16 h later using a multichannel cell harvester (Model ZT-II; Zhejiang, China). The radioactivity of each sample was counted in a liquid scintillation counter (Tri-Carb 2100CA; Packard, San Diego, CA, USA). The mean counts per minute (cpm) of triplicate wells from each mouse was used for statistical calculations and data are expressed as the mean ± SEM (standard error of the mean) cpm incorporated into insoluble DNA minus background.

Statistical analysis

Statistical analysis was performed using the Student *t* test with Bonferroni correction for multiple comparisons, except that where indicated the median end-point titer of IgG in the serum samples was analyzed by Wilcoxon signed rank test. Analyses were performed with Dr. SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL, USA). Differences of $P<0.05$ were taken as statistically significant.

Results

Inactivation of Dct decreases intercellular scavenging capacity for oxidative stress

Levels of intracellular ROS in melanocytes were monitored using an ROS-sensitive probe (H_2DCFDA). The fluorescence intensity increased significantly in Dct-mutant melanocytes pulsed with 100 μM H_2O_2, but ROS levels both in Dct-mutant and in wild-type melanocytes were similar to those of the mock controls (Fig. 1A). Eumelanin pigment granules identified by Fontana–Masson staining were greatly decreased in Dct-mutant melanocytes compared with wild-type melanocytes. There were also very few stage III/IV melanosomes seen in Dct-mutant melanocytes compared with wild-type melanocytes (Fig. 1B). Western blotting and enzyme activity assays showed that the level of the mutant Dct protein was reduced to 30% and its enzyme activity was reduced to 62%, compared with wild-type Dct, but no obvious difference was seen in the protein expression of tyrosinase or Tyrp-1 (Fig. 1C, Table 1). Catalase activity assays also showed that similar activities of catalase were found in both types of melanocytes (data not shown). These results indicate that the *slaty* mutation in Dct decreases eumelanin production, affecting melanosomal maturation and weakening intercellular ROS scavenging.

Oxidative stress induces immune hyperresponsiveness to late-stage melanosomal proteins but not to early-stage melanosomal proteins

Sucrose density-gradient ultracentrifugation was utilized to prepare highly purified melanosomal components (Fig. 2A). Ultrastructural analysis of the various density fractions from the sucrose gradients revealed that the 1.6 and 1.8 M sucrose fractions contained essentially stage III/IV melanosomes and the 1.2 and 1.4 M sucrose fractions contained mostly stage I/II melanosomes (Fig. 2B). There were very few melanized melanosomes seen in the dense fractions of Dct-mutant melanocytes compared with wild-type melanocytes. Western blot analysis revealed that the three melanogenic proteins were similarly abundant in the dense or less dense parts of the gradient except that Dct protein was reduced obviously in fractions containing late-stage melanosomes derived from Dct-mutant melanocytes (Fig. 2C and Table 1). VADC1/porin protein (a mitochondrial marker) was similarly distributed in each of the isolated melanosomal fractions (data not shown).

To gain more insight into the antioxidative role of melanin pigment in maintaining the immune hyporesponsiveness to melanosomal proteins, proteins of late-stage melanosomes (melanin-abundant) and

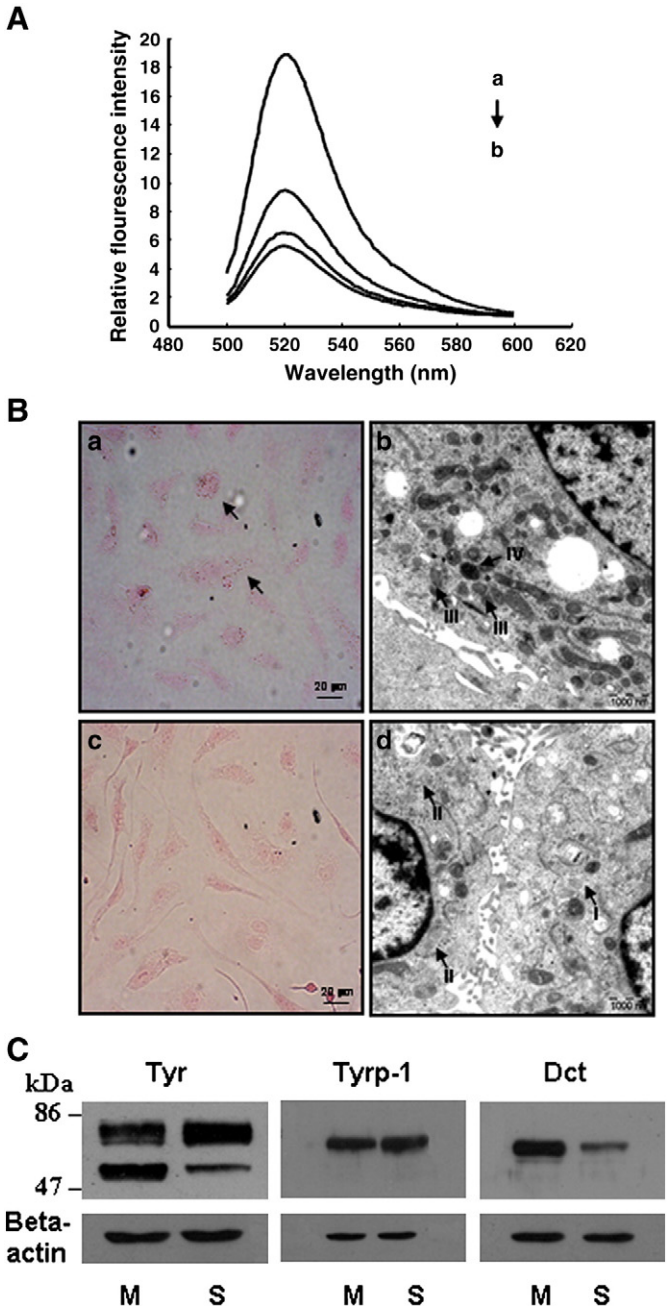

Fig. 1. Comparative analysis of intracellular ROS levels, pigmentation, and ultrastructure, and the expression profiles of melanogenic proteins between melan-a and *slaty* melanocytes. (A) ROS levels were measured using H_2DCFDA labeling. The fluorescence intensity of DCF was read using a spectrofluorimeter and indicates the mean concentration of ROS in the tested cell suspension. (a) *slaty* cells pulsed with 100 μM H_2O_2 for 1 h; (b) melan-a cells pulsed with 100 μM H_2O_2 for 1 h; (c) mock control for *slaty* cells; (d) mock control for melan-a cells. (B) Visualization of the extent of pigmentation between both types of melanocytes using Fontana–Masson staining (a and c, scale bar, 20 μm) or transmission electron microscopy (b and d, scale bar, 1000 nm). Numerous coarse brownish melanin granules (a, arrowheads) and stage III/IV melanosomes (b, arrowheads) are seen in melan-a cells. In contrast, few melanin granules (c) and many stage I/II melanosomes (d, arrowheads) are visible in *slaty* cells. (C) Equal amounts of protein (10 μg) were electrophoretically separated on 10% SDS–PAGE gels and were transferred to PVDF membranes. Specific bands of tyrosinase, Tyrp-1, and Dct were detected with αPEP7 (1:1000), αPEP1 (1:2000), and αPEP8 (1:2000) rabbit polyclonal antibody, respectively. β-Actin served as a loading control. Numbers on the left are molecular weight standard proteins (in kDa). M, melan-a cell lysate; S, *slaty* cell lysate.

of early-stage melanosomes (melanin-scarce) were purified from H_2O_2-pulsed melanocytes and were then used to immunize and boost CB6F1 mice for in vivo immune induction according to our well-established protocol [25,26]. As shown in Fig. 3, the immunized mice exhibited hyperresponsiveness to a challenge with late-stage melanosomal proteins compared with early-stage melanosomal proteins, as measured by T cell proliferation and antimelanosomal protein IgG titers, especially to late melanosomes derived from Dct-mutant melanocytes. These results show that oxidative stress (H_2O_2-pulse treatment) significantly increased the immunogenicity of late melanosomal proteins, and this was most pronounced for proteins from Dct-mutant melanocytes.

Synthetic DHICA-rich melanin confers antioxidative protection to late melanosomal proteins

It is well known that the *slaty* mutation dramatically decreases the protein level and enzymatic activity of Dct [12,18], and thereby melanin with a lower DHICA/DHI ratio that shows insufficient scavenging capacities for ROS is produced by Dct-mutant melanocytes [10]. Next, we synthesized DHI–melanin and DHI/DHICA (1:1)–melanin using tyrosinase-catalyzed oxidation of their monomers [24] and then determined the antioxidative protection of both types of melanins on purified late-stage melanosomes in vivo. As shown in Fig. 4, late-stage melanosomal proteins from Dct-mutant melanocytes were incubated with exogenous DHI/DHICA (1:1)–melanin or DHI–melanin and then were pulsed with 100 μM H_2O_2. These oxidatively stressed proteins were then used to immunize and boost CB6F1 mice. T cell proliferation and IgG antibody responsiveness to these melanosomal proteins increased dramatically in mice challenged with late-stage melanosomal proteins incubated with DHI–melanin, but not with DHI/DHICA (1:1)–melanin. These results show that exogenous DHICA-rich melanin provides a potent antioxidative protection against ROS to maintain immune hyporesponsiveness to melanosomal proteins.

Oxidative stress induces immune hyperresponsiveness to late melanosomal proteins derived from Dct-mutant but not from wild-type melanocytes

Based on the above results, we designed an experiment to further verify whether melanosomal proteins from Dct-mutant melanocytes are more susceptible than those of wild-type melanocytes to oxidative modification. First, we purified late-stage melanosomal proteins from the denser sucrose fractions of both types of melanocytes in the presence or absence of H_2O_2-pulse treatment and then used them to immunize and boost CB6F1 mice to compare their ability to induce an immune response. As shown in Fig. 5, both T cell proliferation and IgG antibody responsiveness to melanosomal proteins were enhanced significantly in mice challenged with late-stage melanosomal proteins derived from Dct-mutant melanocytes, but not from wild-type melanocytes, after pulse treatment with H_2O_2. These results show that the mutation in Dct results in the loss of antioxidative protection to maintain immune tolerance of melanosomal proteins.

Discussion

Despite the fact that compartmentalization of the melanogenesis process represents a strategy to confine toxic *o*-quinonic intermediates and ROS generated during melanin biosynthesis to the membrane-bound melanosomes, melanocytes also need to possess either a potent enzymatic or a nonenzymatic defense mechanism to scavenge oxidative stress in a timely fashion, further ensuring their viability [10]. As the main substances filling the bulk of melanosomes, natural eumelanins are heterogeneous polymers containing monomeric units derived both from DHICA and DHI, the product of the spontaneous decarboxylation of dopachrome [28,29]. The relative proportions of DHICA-derived carboxylated monomers and DHI-derived decarboxylated monomers vary widely from one type of natural pigment to another, conferring the resulting eumelanin with diverse antioxidative capacities [10,30].

Table 1
Relative expression and enzyme activity of melanogenic proteins in the whole-cell lysates and the melanosomal fractions.

	Melan-a MCs			*slaty* MCs		
	Whole-cell lysates	Early MS	Late MS	Whole-cell lysates	Early MS	Late MS
Enzyme activity (% of control)						
Tyrosinase	100.0	130.8 ± 6.0	130.6 ± 13.0	102.6 ± 1.0	135.8 ± 7.0	137.7 ± 2.0
Dct	100.0	99.8 ± 2.0	97.9 ± 2.0	62.1 ± 2.0	54.1 ± 2.0	28.0 ± 2.0*
Protein expression						
Tyrosinase	1.0	1.1	1.0	1.0	1.1	1.1
Tyrp-1	1.0	0.8	0.9	1.0	0.9	0.9
Dct	1.0	0.8	0.8	0.3	0.3	0.1*

Enzyme activity of tyrosinase and Dct was estimated using spectrophotometric assays as described in the text. The relative activities of tyrosinase and Dct in the whole-cell lysates, early-stage melanosome fractions (early MS) and late-stage melanosome fractions (late MS) are expressed as a percentage of the mean ± SD compared with the respective control, and the whole-cell lysate of melan-a cells is set at 1.0. The protein expression levels of tyrosinase, Tyrp1, and Dct in the whole-cell lysates, early MS, and late MS were determined by Western blotting (Figs. 1 and 2). Immunoreactive bands were quantitated by a densitometric measurement and are expressed as the ratio of each band density to the corresponding loading control (β-actin) band density. Each value represents the mean of two determinations and considers the whole-cell lysate of melan-a cells as 1.0.
*$P<0.001$ versus wild-type melan-a cells.

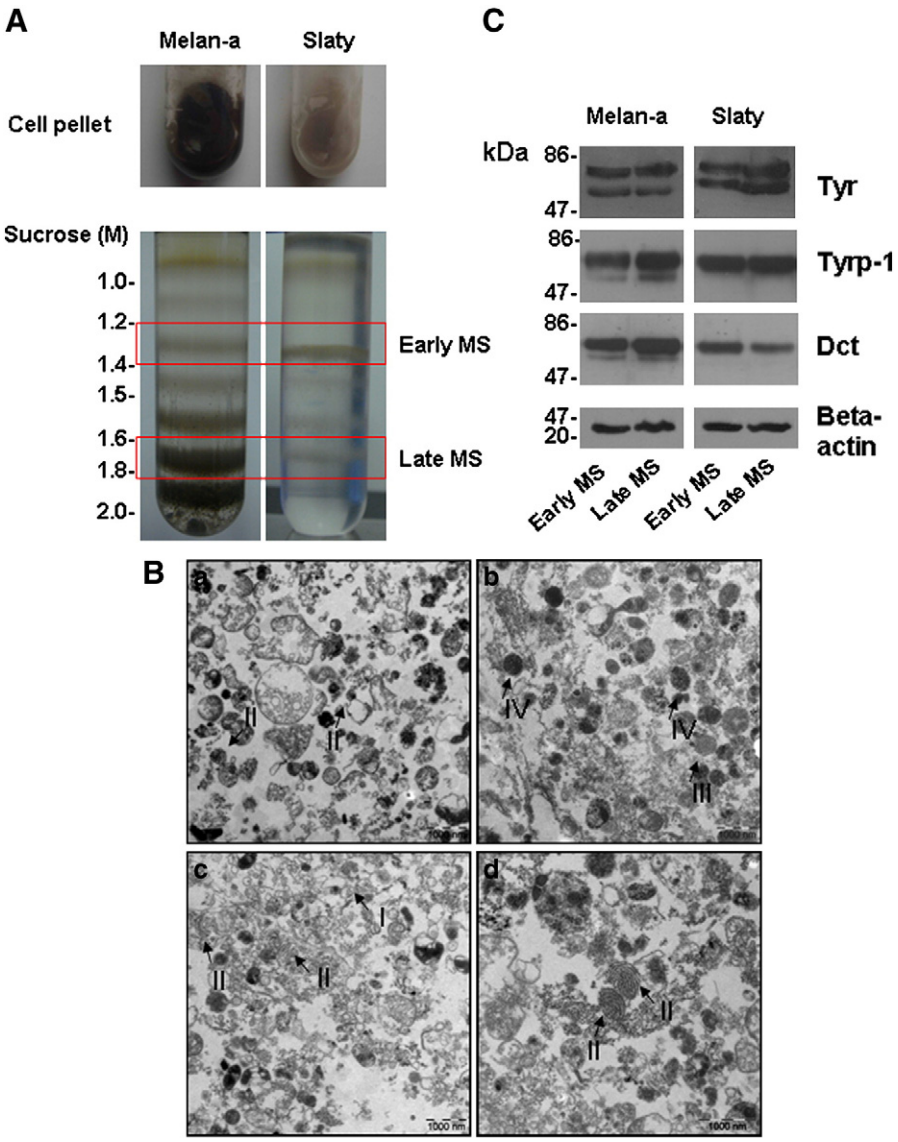

Fig. 2. Purification of early- and late-stage melanosomes by sucrose density-gradient ultracentrifugation. (A) Confluent monolayers of melan-a or *slaty* cells were harvested by trypsinization. The cell pellets varied in color from dark brown (melan-a cells) to milky yellow (slaty cells) and were homogenized on ice using 20 strokes of a Dounce glass–glass homogenizer. Each cellular homogenate was layered on a discontinuous gradient of 1.0, 1.2, 1.4, 1.5, 1.6, 1.8, and 2.0 M sucrose. The melanosomal fractions at various stages were separated as described under Materials and methods. Early- and late-stage melanosomes were recovered from the 1.2–1.4 M and from the 1.6–1.8 M sucrose interfaces, respectively. (B) Enriched melanosome fractions were subjected to ultrastructural analyses for validating melanosome components. Numerous stage III/IV (late) melanosomes are seen in the 1.6–1.8 M fractions of melan-a cells (b, arrowheads), but only stage II melanosomes are seen in *slaty* cells (d, arrowheads). Coated vesicles and stage I/II (early) melanosomes are seen in the 1.2–1.4 M fractions of both melan-a cells (a) and *slaty* cells (c). Scale bar, 1000 nm. (C) Enriched melanosome fractions were subjected to Western blotting to analyze the abundances of melanogenic proteins. The protocol used was as described for Fig. 1C. Early MS, 1.2–1.4 M sucrose fractions; late MS, 1.6–1.8 M sucrose fractions.

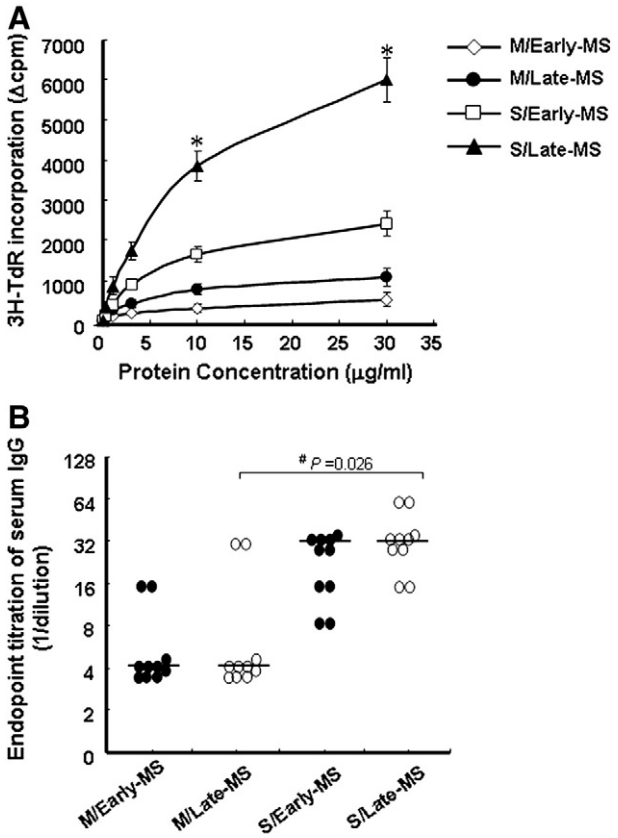

Fig. 3. Determination of immune responses to melanosomal proteins purified from early- or late-stage melanosome fractions in the presence of oxidative stress. (A) T cell responses to melanosomal proteins in mice primed with the proteins as mentioned under Materials and methods. CB6F1 mice were immunized and boosted with early-stage or late-stage melanosomal proteins (M, melan-a cells; S, *slaty* cells). After the boosting, lymph nodes were restimulated with dilutions of the corresponding proteins and pulsed with [^{3}H]thymidine (3H-TdR). Each group contained three to five mice. Results are expressed as means ± SEM of cpm incorporated into insoluble DNA ($n = 2$). *$P < 0.05$ versus the corresponding late-stage counterpart of melan-a cells. (B) Humoral responses to melanosomal proteins in mice primed with the designated proteins. CB6F1 mice were treated as described for (A). After the boosting, sera were collected for testing secondary antibodies to the primed proteins. Antibody IgG titers were assessed by ELISA as the serial dilution of the tested serum to the end point. Sample dilutions were run in duplicate for each mouse; the horizontal line corresponds to the median end-point titer. The Wilcoxon signed rank test was used for comparison of IgG titers in samples. *P* value indicates late-stage melanosomal proteins from the *slaty* cells versus those from the melan-a cells.

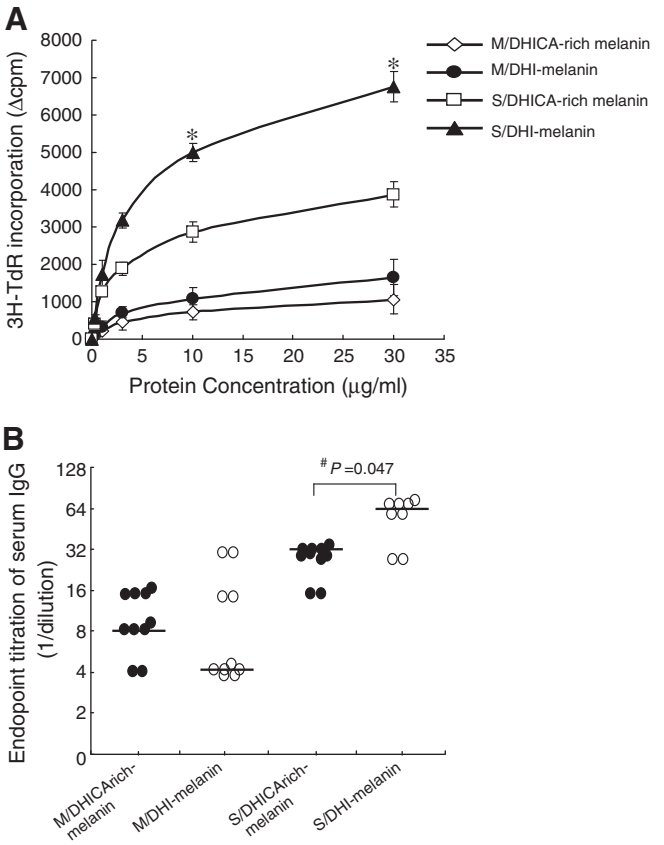

Fig. 4. Determination of immune responses to late-stage melanosomal proteins incubated with synthetic DHICA-rich melanin in the presence of oxidative stress. (A) T cell responses to melanosomal proteins in mice primed with the designated proteins. CB6F1 mice were immunized and boosted with late-stage melanosomal proteins incubated with synthetic DHI/DHICA (1:1)–melanin or synthetic DHI–melanin (M, melan-a cells; S, *slaty* cells). After the boosting, lymph nodes were restimulated with dilutions of the corresponding proteins and pulsed with [^{3}H]thymidine. Each group contained three to five mice. Results are expressed as means ± SEM of cpm incorporated into insoluble DNA ($n = 2$). *$P < 0.05$ versus the corresponding DHICA rich-melanin treatment. (B) Humoral responses to melanosomal proteins in mice primed with the indicated proteins. CB6F1 mice were treated as described for (A). After the boosting, sera were collected for testing secondary antibodies to the primed proteins. Antibody IgG titers were assessed by ELISA as the serial dilution of the tested serum to the end point. Sample dilutions were run in duplicate for each mouse; the horizontal line corresponds to the median end-point titer. The Wilcoxon signed rank test was used for comparison of IgG titers in samples. *P* value indicates late-stage melanosomal proteins incubated with DHI–melanin versus with DHICA–rich melanin.

Our recent study showed that Dct exerts an antioxidative activity by promoting the incorporation of a certain proportion of DHICA monomers into the DHI polymer backbone, by which preferentially DHICA-rich eumelanin is produced [10]. However, relatively little is known about how melanosomal proteins interact with melanin polymers and how melanosomal proteins are protected from oxidative damage, even under conditions of high and/or sustained oxidative stress levels, for maintaining immune hyporesponsiveness toward those proteins [31].

It is widely believed that melanosomes are first created by relatively amorphous and spherical vesicles that bleb from the endoplasmic reticulum and lack tyrosinase activity and any internal structural components (termed stage I melanosomes or premelanosomes). Subsequently, a structural protein (Pmel17/gp100), MART1, and enzymatic proteins (Tyr, Tyrp1, and Dct) are trafficked to stage I melanosomes to induce fusiform fibrillar organelles and to initiate the complex process of melanization (termed stage II melanosomes). Melanin is synthesized and deposited on the internal fibrils (termed stage III melanosomes), and with the further production and deposition of melanin, the melanosomes become homogeneously and highly pigmented with no internal structural detail visible (termed stage IV melanosomes) [32,33]. An interesting ultrastructural observation made by atomic force microscopy is that eumelanin from *Sepia officinalis* is an aggregated structure comprising entities that appear to have diameters on the order of 100 to 200 nm and filaments are seen on much of the surface [34]. Such a structural characteristic of eumelanin gives a hint that the melanin formed in stage III melanosomes is not randomly deposited in the intralumenal proteinaceous matrix, probably contributing to the site where melanogenic proteins reside on the internal fibril architecture [34]. We might further speculate that melanosomal proteins are embedded in the core of each entity in melanosomes, and eumelanin then functions as a shield to protect the entrapped proteins from potential oxidative insults. It is generally accepted that heavily melanized melanosomes are retained within the melanocytes until they are eventually transferred to the surrounding keratinocytes. As keratinocytes undergo terminal differentiation and migrate to the stratum corneum, transferred melanosomes are gradually degraded to release the encapsulated melanin for maximum photoprotection and free

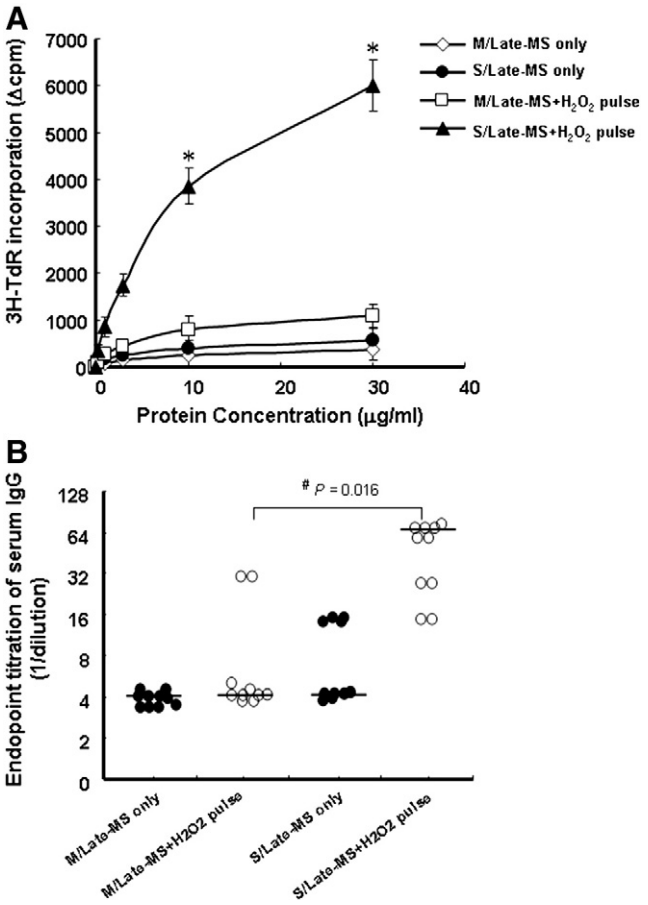

Fig. 5. Determination of immune responses to late-stage melanosomal proteins in the presence or absence of oxidative stress. (A) T cell responses to melanosomal proteins in mice primed with the designated proteins. CB6F1 mice were immunized and boosted with late-stage melanosomal proteins in the presence or absence of 100 μM H_2O_2-pulse treatment (M, melan-a cells; S, *slaty* cells). After the boosting, lymph nodes were restimulated with dilutions of the corresponding proteins and were pulsed with [^{3}H] thymidine. Each group contained three to five mice. Results are expressed as means ± SEM of cpm incorporated into insoluble DNA ($n = 2$). *$P < 0.05$ versus the corresponding *slaty* cell group. (B) Humoral responses to melanosomal proteins in mice primed with the indicated proteins. CB6F1 mice were treated as described for (A). After the boosting, sera were collected for testing secondary antibodies to the primed proteins. Antibody IgG titers were assessed by ELISA as the serial dilution of the tested serum to the end point. Sample dilutions were run in duplicate for each mouse; the horizontal line corresponds to the median end-point titer. The Wilcoxon signed rank test was used for comparison of IgG titers in samples. *P* value indicates late-stage melanosomal protein from *slaty* cells versus melan-a cells.

radical scavenging activity [35]. However, the ratio of keratinocytes to melanocytes in human epidermis is about 36:1, and keratinocytes may act as a major source of H_2O_2 by passive diffusion and thus affect melanocytes and melanosomal proteins, because H_2O_2 is a neutral molecule capable of permeating through cellular membranes [36]. To date there is compelling in vitro and in vivo evidence for epidermal H_2O_2 overproduction in vitiligo. Four potential sources of epidermal H_2O_2 generation in vitiligo have been identified: (1) perturbed (6*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin de novo synthesis/recycling/regulation; (2) impaired catecholamine synthesis with increased monoamine oxidase A activities; (3) low glutathione peroxidase activities; and (4) "oxygen burst" via NADPH oxidase from a cellular infiltrate [37]. Loss of immune tolerance toward melanosomal proteins under conditions of oxidative stress causes autoimmune destruction of melanocytes in vitiligo that might be due to: (1) the oxidative fissure of the indole unit within the melanin polymer by high-density attack of hydroxyl radicals to expose cryptic epitopes of entrapped melanosomal proteins to the immune system [38]; (2) the oxidative modification of inappropriately cleaved melanosomal protein fragments bound to indole molecules that may serve as a hapten [39]; and/or (3) the disruption of DHICA/DHI-derived units in eumelanin by an uncharacterized factor to diminish their antioxidation, even showing a pro-oxidant behavior.

To further explore the early upstream initiating event involved in the oxidative stress-mediated autoimmune response to melanosomal proteins in vitiligo, we utilized a sucrose density-gradient ultracentrifugation protocol to achieve highly enriched melanosomal components from Dct-mutant and from wild-type melanocytes in the presence or absence of H_2O_2-pulse treatment (Fig. 2) and then immunized and boosted CB6F1 mice with such proteins to evaluate their capacities to provoke an immune response. Our findings are consistent with a previous study [18], which suggested that the *slaty* mutation in mice significantly decreases DHICA-rich eumelanin production, blocks melanosomal maturation at stage III, and lessens intercellular ROS scavenging (Fig. 1). To explore the role of melanin pigment in maintaining the immune hyporesponsiveness to melanosomal proteins, we first determined whether the melanosomal proteins derived from early- or from late-stage fractions might elicit the different immune response because early-stage melanosomes contain much less melanin than late-stage melanosomes. As shown in Fig. 3, mice exhibited hyperresponsiveness to a challenge with late-stage melanosomal proteins, especially to late-stage melanosomal proteins derived from Dct-mutant melanocytes. This result implies that the eumelanin with altered proportions of DHICA- and DHI-derived units in Dct-mutant melanocytes exerts a pro-oxidant activity instead of an antioxidant activity. To further verify this conjecture, we synthesized DHI–melanin and DHI/DHICA (1:1)–melanin, incubated them with late-stage melanosomal proteins in vitro, and then treated them with micromolar doses of H_2O_2 to determine their antioxidative protection. As we expected (Fig. 4), T cell proliferation and IgG antibody responses were significantly induced by late-stage melanosomal proteins incubated with DHI–melanin, but not with DHI/DHICA (1:1)–melanin.

Another view, which does not come directly within the scope of the present study, concerns whether immune responses similar to those in vitiligo may operate in the destruction of malignantly transformed melanocytes [40]. Intense effort has been devoted to the deliberate induction of specific autoimmune responses against melanosomal proteins, such as Tyr, Tyrp1, Dct, gp100/Pmel17, and MART1/melan-a, to achieve anti-tumor effects through vaccine strategies [41]. Unfortunately, such "self" melanosomal proteins are generally nonimmunogenic because self-reactive T cells have been physically or functionally deleted in the thymus or in the periphery. Albeit not yet defined, our data show that melanosomal proteins derived from Dct-mutant melanocytes are more susceptible than those from wild-type melanocytes to oxidative stress (Fig. 5). One possible interpretation is that the mouse *slaty* mutation, a single amino acid substitution (R194Q(Arg194→Gln)) that occurred spontaneously in the first metal-binding domain of the Dct protein, seriously affects the steric configuration of the mutant Dct protein and the stability of the melanogenic protein complex [12,18]. So far, no polymorphisms or mutations in the human DCT gene have been reported to correlate with vitiligo susceptibility, let alone its pathogenesis. Alonso et al. [42] used Affymetrix U133a version 2.0 gene expression microarrays to screen for polymorphisms within 4 to 5 kb of the proximal regulatory regions of three melanogenic loci (tyrosinase, Tyrp1, and Dct) in nine melanocyte cell lines (five from lightly pigmented donors and four from darkly pigmented donors) plus their respective unirradiated controls. The findings of that study were that nine SNPs plus one indel (involving 1 bp) in the 4.1-kb region upstream of the Dct initiation point are responsible for skin pigmentation difference among Africans, Europeans, and Asians. Dct and Tyrp1 showed higher haplotype diversity in the African populations analyzed, whereas tyrosinase showed similar levels of haplotype diversity across populations [42]. Kingo et al. [43] measured the

mRNA expression profiles of eight genes from the melanocortin system and two genes involved in melanogenesis (Tyrp1 and Tyrp2/Dct). RNA was extracted from both lesional and nonlesional skin of vitiligo patients and in non-sun-exposed skin of healthy subjects. The results showed that the Dct and Tyrp1 genes are down-regulated in lesional skin compared to nonlesional vitiligo skin or skin of healthy controls, whereas they are significantly up-regulated in uninvolved vitiligo skin compared to healthy control samples [43]. Evidence also exists for a ratio imbalance of pheomelanin vs eumelanin in the depigmented lesions, especially in active vitiligo patients [44]. At this point, we are uncertain whether vitiligo melanocytes also have a ratio disorder of DHICA– vs DHI–melanin. It is critically important to be able to determine accurately that DHICA– or DHI–melanin is altered in vitiligo lesions, thereby explaining the near-exclusive role of DHICA–melanin in antioxidative protection against melanocytes. The correlation of potential molecular lesion and dysfunctional regulation of the Dct gene with vitiligo pathogenesis deserves further careful study and analysis.

Taken together, these data provide unequivocal evidence of the critical role of Dct in regulating the antioxidative capacity of eumelanin by changing the proportion of DHICA monomer incorporated into the DHI polymer backbone and in maintaining immune hyporesponsiveness to melanosomal proteins by DHICA-mediated antioxidation. The altered form of melanosomal proteins due to oxidative modification is a prerequisite for breaching immune tolerance and eliciting a response against these proteins. Although it is difficult to characterize the specific antigenic determinants among melanosomal proteins, we feel that it is important to purify each recombinant protein from a mammalian expression system to test its immune induction in our murine system, which holds substantial promise for better defining the mechanism of vitiligo pathogenesis and for devising a whole-cell vaccine targeting immunodominant melanosomal antigens against melanoma [45].

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