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HSP70i Accelerates Depigmentation in a Mouse Model of Autoimmune Vitiligo

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Abstract

Vitiligo is a T-cell-mediated autoimmune disease of the skin. Progressive depigmentation accelerates in response to stress. Personal trauma, contact with bleaching phenols, overexposure to UV, and mechanical injury can lead to progressive loss of melanocytes. This study was focused on the role of stress protein heat shock protein (HSP)70 for translating stress into an autoimmune disease to melanocytes. Intracellular HSP70 can act as a cytoprotectant, preventing apoptosis in cells under stress. Isoform HSP70i can be secreted by live cells, and in prior *in vitro* studies, HSP70 has been shown to activate dendritic cells and elicit an immune response to chaperoned proteins and peptides. Here, the role of HSP70 in precipitating and perpetuating vitiligo was assessed *in vivo* in a mouse model of autoimmune vitiligo. In this model, depigmentation was introduced by gene gun vaccination with eukaryotic expression plasmids encoding melanocyte differentiation antigens. Inclusion of human and mouse-derived inducible HSP70 in the vaccination protocol significantly increased and accelerated depigmentation in this model, accompanied by the induction of prolonged humoral responses to HSP70. Cytotoxicity toward targets loaded with a K(b)-restricted tyrosinase-related protein 2-derived peptide correlated with depigmentation. The data presented strongly support a role for HSP70i in progressive depigmentation *in vivo*.

INTRODUCTION

Vitiligo patients present with progressive depigmentation involving progressive loss of melanocytes from the skin (Le Poole *et al*., 1993a). Loss of melanocytes within hair follicles has also been reported, and premature graying as well as hair loss appear to mechanistically associate with vitiligo (Hedstrand *et al*., 2006).

The process of depigmentation is associated with a T-cell-mediated immune response to melanocytes during disease progression (Ogg *et al*., 1998). This etiological factor was long overlooked, as T-cell infiltrates are not observed in stable disease and immune infiltrates are restricted to a narrow margin of skin coinciding with the disappearance of melanocytes (Das *et al*., 2001).

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This study was conducted at Oncology Institute, Loyola University Chicago, Maywood, IL, USA.

CONFLICT OF INTEREST

ICLP has held a collaborative Research Agreement with Borean Pharma in Aarhus, Denmark.

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In vitiligo, a wide array of stress factors can provoke an autoimmune response to melanocytes. Precipitating factors in vitiligo include overexposure to UV (sunlight) during a sunny vacation, and contact with bleaching phenols, including 4-tertiary butyl phenol (4-TBP) in the home or workplace, as in occupational vitiligo (Boissy and Manga, 2004; Namazi, 2007). Emotional stress is similarly reported as a precipitating factor by patients, including death of first-degree relatives and childbirth (Barisi -Drusko and Rucevi , 2004). Mechanical injury after cuts and burns can induce new lesions, and 50% of patients report observing Koebner's phenomenon at the sites of minor scrapes, cuts, and burns to the skin (unpublished observation).

A hereditary component to vitiligo is evident from the observation that only 0.5–1% of individuals will develop progressive depigmentation of the skin in response to stress (Spritz, 2007). Only a subset of individuals will develop vitiligo in response to stress, suggesting the involvement of a hereditary component.

It has not yet been elucidated how exposure to stress in individuals with a genetic propensity to develop vitiligo translates into an autoimmune disease targeting melanocytes within the skin. *In vitro* studies have implicated heat shock protein (HSP)70 as a precipitating factor in vitiligo. Specifically, melanocytes overexpress HSP70 in response to bleaching phenol 4-TBP (Kroll *et al.*, 2005). Increased amounts of HSP70 were detectable in the supernatant of vitiligo compared to control melanocytes (Kroll *et al.*, 2005). Exposure to 4-TBP sensitized melanocytes to dendritic cells (DC)-mediated cytotoxicity, mediated at least, in part, by enhanced expression of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) by HSP70-exposed DCs and elevated expression of TRAIL receptor 1 and TRAIL receptor 2 by melanocytes. HSP70 enhances antigen uptake and processing by DC, activating DC, and stimulating T-cell-mediated immune responses (Haug *et al.*, 2005; Bendz *et al.*, 2007; Pido-Lopez *et al.*, 2007). Consistent differential expression of HSP70 between non-lesional and lesional skin of three vitiligo patients as observed by immunohistology infers a role for HSP70 in depigmentation (Le Poole and Luiten, 2008), and the stress protein has been implicated in autoimmune responses *per se* by others (Multhoff, 2006).

HSP70 is one of several stress proteins upregulated in response to heat shock (Bivik *et al.*, 2007). Under stress, cell shut down overall protein synthesis while maintaining stress protein expression. HSPs will chaperone proteins to protect the cell from undergoing apoptosis until the stressful episode has passed (Bivik *et al.*, 2007). HSPs chaperoning proteins and peptides from dying cells can thus induce an immune response to cells from which they were derived.

The stress protein HSP70 is unique in the respect that it was shown to be secreted by live cells (Mambula *et al.*, 2007). Whereas the process of HSP70 secretion remains poorly understood, it appears to involve exosome formation by tumor cells (Gastpar *et al.*, 2005). Secretion was first reported for neuronal cells, understandable from the standpoint of synaptic vesicles projected into the extracellular under normal physiologic conditions. Melanocytes may similarly secrete HSP70 containing melanosomes under stress. This is particularly credible because HSP70 secretion is associated with lysosomes (Mambula and Calderwood, 2006). Intracellular HSP70i (inducible HSP70) translocates to lysosomes and lysosomes are highly congruent with melanosomes, suggesting that HSP70 can localize to melanosomes as well (Le Poole *et al.*, 1993b). As melanosome export is a process important to skin pigmentation, export of HSP70 chaperoning peptides and proteins from the melanosomal compartment can likely contribute to the immunogenicity of melanocyte differentiation antigens homing to the melanosomal organelle.

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Under standard physiologic conditions, constitutive HSP70 isoforms are expressed within the cell offering cytoprotection to the cell. A total of 11 genes have been identified coding for HSP70 isoforms with a high degree of homology (Tavaria *et al.*, 1996), sharing molecular properties including an approximate molecular weight of 70 kDa and an N-terminal peptide binding domain regulated by a C-terminal ATPase domain. When released into the extracellular milieu through cell death or active secretion, HSP70 interacts with DC to enhance uptake of antigens. HSP70 induces DC maturation, and the term ‘chaperokine’ has been coined to describe its role in immune stimulation (Asea, 2006). These properties render HSP70 an ideal vaccine adjuvant for antitumor treatment, stimulating immune reactivity to the cells from which they are derived. Clinical trials for tumor-derived HSP-based vaccines are underway (Trimble *et al.*, 2003).

Following experimental evidence suggesting for a role of HSP70 in depigmentation *in vitro*, the challenge has been to develop a reproducible *in vivo* mouse model of autoimmune vitiligo. The current model makes use of vaccination strategies otherwise used to test therapeutic efficacy against melanoma tumors. It has been observed that progressive depigmentation of the pelage can be achieved by gene gun vaccination with DNA encoding melanocyte differentiation antigens, which is the premise on which the current model is built (Overwijk *et al.*, 1999).

Human TRP-2, as well as human and mouse-derived HSP70i were cloned into eukaryotic expression vectors. It should be noted that TRP-2 serves as a model antigen, whereas several other melanocyte differentiation antigens can similarly be included in the vaccine to develop a functional model of autoimmune vitiligo. Xenogeneity of melanocyte differentiation antigens has been shown to contribute to their immunogenicity (Engelhorn *et al.*, 2006). Mice were gene gun vaccinated and depigmentation was scored by scanning anesthetized mice. Relevant observations regarding the extent of depigmentation were correlated with the assessment of the humoral and cellular responses to vaccine components. The *in vivo* observations in this mouse model are important for establishing a potential role for HSP70 in precipitating and expansion of progressive autoimmune vitiligo.

RESULTS

Depigmentation in response to gene gun vaccination

In Figure 1a, depigmentation is shown in 129S7 agouti mice that were gene gun vaccinated in the flank (ventral coloration is off-white to yellow in this mouse strain). Groups of five mice are shown, each representing 10 mice vaccinated with either empty vector control plasmid, a plasmid encoding human HSP70 (hHSP70), a plasmid encoding hTRP-2, or a combination of HSP70 and TRP-2-encoding plasmids. All groups were vaccinated with the same total amount of DNA. The only group of mice that displayed visible depigmentation in this experiment was the group vaccinated with a combination of hTRP-2 and hHSP70. Six weeks after the final vaccination, depigmentation in this group was significantly increased at $P<0.05$ compared with mice vaccinated with control vector DNA ($P=0.03$) or from mice vaccinated with hTRP-2 alone ($P=0.04$). In Figure 1b, data are shown for C57BL/6 mice vaccinated with the same plasmids in the ventral region. These data were consistent with those observed for 129S7 mice. Whereas some depigmentation was observed in response to hTRP-2 alone 1 month after the final vaccination, significant and persistent depigmentation was observed only in the mice vaccinated with a combination of hTRP-2 and hHSP70 plasmids.

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Comparing the depigmenting effects of hHSP70 versus mHSP70

In Figure 2a, results are shown for C57BL/6 mice that were vaccinated with the same plasmids as those included in Figure 1, as well as with mouse-derived mhsp70A1A-encoding plasmid alone or in combination with hTRP-2 encoding DNA, all at the same final DNA concentrations. In this figure, it can be observed that both mouse- and human-derived HSP70 accelerate autoimmune depigmentation to the same extent. No significant difference was detected among groups vaccinated with hTRP-2 combined with either hHSP70i or mhsp70A1A 6 weeks after the final vaccination. In Figure 2b, data are shown for dorsal depigmentation, followed away from the original vaccination site in C57BL/6 mice. Mouse scans (insets) illustrate the data represented in the graph, with significant dorsal depigmentation observed only in mice vaccinated with a combination of hTRP-2 and mhsp70-encoding plasmids.

Immunohistology of depigmented skin

Figure 3 depicts the skin of depigmented (hTRP-2 and hHSP70 vaccinated) versus normally pigmented (control vector vaccinated) mice. Immunohistology of frozen skin sections was performed to detect the presence and location of melanocytes and T cells. TRP-2 and CD3ε immunostaining was highlighted by arrows in samples of control vector-vaccinated skin. It was consistently observed that melanocytes were absent from follicles of depigmented hair. T cells that were easily detectable in skin surrounding pigmented hair follicles, and abundant within mouse epidermis of control vector-vaccinated skin samples, were virtually absent from skin with depigmented hair.

Humoral responses to gene gun vaccination

In Figure 4, blots probed with sera from mice vaccinated with hTRP-2 (a), control vector (b), or hHSP70 alone (c), or a combination of hHSP70 and hTRP-2 (d) versus a combination of mHSP70 and hTRP-2 (e) are shown to illustrate a prominent band observed with a molecular weight of approximately 70 kDa exclusively in mice vaccinated with (a combination of) expression vectors that encode HSP70 as shown in panels c–e. Conversely, mice vaccinated with TRP-2 or empty vector (a, b) DNA alone did not mount a prominent humoral response to a 70 kDa protein. A lack of humoral responses to TRP-2 after vaccination with any of the genes included in our vaccine strategies, including hHSP70, mHSP70, or TRP-2, was illustrated by a lack of reactivity with TRP-2-transfected COS cells in Figure 4c and e. Finally, in Figure 4f, the recognized 70 kDa band was identified as HSP70, loaded as a purified protein versus an equal amount of HSP60 in the adjacent lane.

Cytotoxicity to a major histocompatibility complex class I restricted, TRP-2-derived peptide

Figure 5 illustrates reactivity to a K(b)-restricted, TRP-2-derived peptide major histocompatibility complex class I-restricted peptide (peak P7) to illustrate cytotoxic T-cell responses to melanocytes induced by vaccines containing a combination of HSP70 and TRP-2-encoding plasmids. Cytotoxicity amounting to 30.1% lysis in this group was observed toward spleen cells loaded with a TRP-2-derived peptide within 18 hours (Figure 5b). Such reactivity was not observed in mice vaccinated with HSP70 alone (Figure 5b). For these experiments, mice were re-vaccinated once 5 days before a challenge with labeled and loaded spleen cells from syngeneic mice.

DISCUSSION

Gene gun vaccination has proven a successful means of introducing antitumor vaccines into mice to assess their efficacy in preventing or treating tumor outgrowth (Lauterbach *et al.*,

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2006). In particular, this mode of vaccine administration has been used to demonstrate efficacy of vaccines to treat melanoma (Steitz *et al.*, 2000). DNA-based vaccination against differentiation antigen TRP-2 was shown to induce measurable antitumor responses when using human-derived sequences. When administering antimelanoma vaccines, reduced tumor growth was found to be associated with pelage depigmentation in mice, reminiscent of leucoderma observed in melanoma patients developing immune responses to their tumor. Indeed, leucoderma is considered a positive prognostic factor among melanoma patients (Nordlund *et al.*, 1983). An important observation has been the development of pelage depigmentation in the absence of tumor involvement among vaccinated mice (Nordlund *et al.*, 1983). It has since been observed that stress introduced by a needle prick can precipitate depigmenting lesions in TRP-2-vaccinated mice (Lane *et al.*, 2004). Such observations indicate that melanocyte differentiation antigen-based vaccination of mice can reproduce a condition very reminiscent of progressive human vitiligo. We have exploited this principle as a model to study precipitating factors for vitiligo, a much needed development in this line of research where successful mouse models are lacking.

In this study, the main objective has been to demonstrate that HSP70, a stress protein more actively secreted by vitiligo than control melanocytes (Kroll *et al.*, 2005), can activate an immune response to melanocytes in our *in vivo* gene gun vaccination model of human vitiligo. In initial experiments (data not shown), it became clear that high-dose vaccination with DNA encoding melanocyte differentiation antigens alone can introduce depigmenting lesions of the pelage among vaccinated mice, consistent with earlier observations. Given our interest in establishing a role for HSP70 in autoimmune depigmentation within a mouse model of human vitiligo, vector constructs were generated encoding the human (and mouse) HSP70 molecules(s). Once introduced into mice, the HSP70 molecule proved to be an efficient instigator of depigmentation among treated mice in two separate models assessed, when administered in combination with suboptimal doses of 2.4 μg DNA encoding a target antigen of interest. At this concentration, TRP-2-encoding plasmid alone did not induce significant depigmentation. The mechanism of gene gun vaccination has been ascribed, in part, to direct introduction of the DNA into Langerhans cells and DCs (Lauterbach *et al.*, 2006). Given the protein chaperoning function of stress proteins, any cell upregulating expression of the TRP-2 and HSP70 genes can become an efficient source of antigen to be subsequently taken up and cross-presented by DCs, further contributing to the antigenicity of this particular vaccine. In a pilot experiment, results indicated that HSP70 can similarly enhance gp100-induced depigmentation (data not shown).

HSP70 has caught attention in reference to autoimmune vitiligo in the past (Kroll *et al.*, 2005). In this regard, the stress protein is of interest because it can be secreted by live cells. Initial observations supported differential expression of the stress protein among non-lesional and lesional skin of vitiligo patients (unpublished observation). Stress proteins, in general, and HSP70, in particular, are of interest as they protect cells from undergoing apoptosis in crisis situations by chaperoning proteins and peptides within the cell. Combined with DCs activating properties, such as enhanced uptake of antigens and enhanced expression of DC activation markers in response to HSP70 exposure, the stress protein may be responsible for translating stress into an autoimmune response to melanocytes in vitiligo. This study strongly supports this hypothesis. The data show that human as well as mouse-derived HSP70 can accelerate an immune response to melanocytes in mice. Interestingly, development of depigmentation at sites distant from the original vaccination site was restricted to the mice vaccinated with (combinations of) mouse-derived HSP70. It should be noted that this differential fact was observed under conditions where the amount of antigen-encoding DNA was limiting (2.4 μg weekly for 4 weeks) and that dorsal depigmentation has been routinely observed in mice vaccinated with 4 μg of target antigen-encoding DNA alone (unpublished observation). The restricting experimental conditions used here highlight the

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superior depigmenting effect of mouse-derived HSP, possibly explained by the biological function, rather than the immunogenicity, of HSP70 carrying responsibility for its immune activating properties.

Mice vaccinated with either human or mouse HSP70 elicited a humoral response to the protein with circulating antibodies (Abs) readily detectable 3 months after vaccination (later time points not tested). By contrast, Abs to TRP-2 were never found in TRP-2-vaccinated mice. Although the presence of Abs reactive with TRP-2 in vitiligo sera has been reported (Okamoto *et al*., 1998), such Abs likely represent an epiphenomenon that occurs when TRP-2 is spilled from dying melanocytes. As the protein is contained in the melanosomal compartment of living cells, Abs to TRP-2 are unlikely to affect melanocyte viability or contribute to depigmentation. Abs to mouse HSP70 are possibly a better "fit" for the naturally expressed protein to enhance its uptake (and its chaperoned proteins) by DCs, explaining why rapid depigmentation of the dorsal area was observed solely in mHSP70-vaccinated mice.

Importantly, whereas depigmentation was observed in mice with Ab titers to HSP70 outlasting progressive pelage depigmentation, the development of vitiligo was associated with a loss of melanocytes (Figure 3a), a re-distribution of resident skin T cells (Figure 3b) and a cytotoxic T-cell response to melanocytes as demonstrated by TRP-$2_{180–188}$-restricted responses during active depigmentation. These findings are well aligned with observations previously reported for human vitiligo skin (Le Poole *et al*., 1993a; Das *et al*., 2001). Cytotoxic T-cell responses were not detectable 3 months or even 6 weeks after vaccination by either ELISA, ELISPOT (enzyme-linked immunosorbent spot), cytotoxicity assays, or dimer staining followed by FACS analysis (results not shown).

These data are best explained by HSP70 facilitating antigen uptake by DCs, thereby facilitating the recruitment of CD4+ and particularly CD8+ T cells toward the skin. A direct cytolytic effect of HSP70-reactive Abs toward mouse melanocytes was less likely to contribute to pelage depigmentation, as preliminary data indicated that pooled sera from HSP70-vaccinated mice did not mediate complement-mediated lysis of 4-TBP exposed, stressed mouse melanocytes, or melanoma cells *in vitro* (data not shown). Several receptors for HSPs have thus far been identified, including CD91, CD40, and TLR-2, and TLR-4 (Sanchez-Perez *et al*., 2006). Among these receptors, expression of CD91 was found to be abundant among cells in close proximity to the epidermis and with dendritic morphology in perilesional skin (data not shown). Stress-induced overexpression of stress proteins, particularly HSP70, which can be secreted by live cells and facilitates immune responses by enhancing DC activation, can be a relevant mechanism to explain how stress to the skin translates into an immune response targeting melanocytes. Further because HSP70 is overexpressed by cells in crisis, including melanocytes (Bivik *et al*., 2007), it is well conceivable that melanocytes become prime targets of the immune response following stress, leading to the development of progressing vitiligo lesions. In conclusion, the data provide support for the concept that vitiligo is a T-cell-mediated autoimmune disease that precipitates in response to stress.

MATERIALS AND METHODS

Cloning and sequencing of hTRP-2, hHSP70, and mHSP70

For hTRP-2 expression cloning, RNA was isolated from M14 human melanoma cells (25). TRP-2 transcripts were amplified in the presence of the following primers: 5′-CACCATGAGCCCCCC TTTGGTGGGGGTTTC-3′ (forward) and 5′-CTAGGCTTCTTCTGTG TATCTCTTG-3′ (reverse). The CACC sequence in the upstream primer allowed for directional TOPO cloning of the PCR product into pcDNA3.1D/V5-His-

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TOPO (Invitrogen, Carlsbad, CA). Human HSP70i was amplified from human primary keratinocyte RNA in the presence of primers 5′-ATGGCCGCGGCGATCG-3′ (forward) and 5′-CTAATCTACCTCAATGGTG-3′ (reverse), and mouse HSP70A1A was amplified from mouse skin tissue-derived RNA using primers 5′-ATGGCAAGAACACGGCGATCGGCAT-3′ (forward) and 5′-CTAA TCCACCTCCTCGATGGTGGGTCC-3′ (reverse). HSP70-encoding genes were cloned into pcDNA3.1/CT-GFP-TOPO (Invitrogen). Reverse transcription PCR conditions for all amplifications were as follows: 5 μg RNA was combined with 1× first strand reverse transcription buffer in presence of 1mM each of dNTPs, 10mM DTT (dithiothreitol), 3.3mM $MgCl_2$, 25ngml^{-1} oligodT primer and 200U Supercript II reverse transcriptase at 42 °C, terminating the reaction by heating to 70 °C. Ten percent of the reverse transcription reaction was PCR amplified 1× PCR buffer, 2mM $MgCl_2$, 400 μM each of dNTPs, 0.8 μg ml^{-1} primers and 5U Taq polymerase. In the case of hTRP-2, Taq polymerase was replaced by 2.5U AccuPrime enzyme (Invirtrogen) and additives were replaced by 1× AccuPrime mix (Invitrogen). PCR reactions were run for 40 cycles at 95 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 100 seconds, followed by 10 minutes at 72 °C. PCR products were cloned into the appropriate vectors according to the manufacturer's instructions. Four bacterial colonies from each cloning procedure were subjected to restriction analysis, and a clone containing the gene in the correct orientation was used for a MegaPrep endotoxin-free isolation procedure (Qiagen, Valencia, CA) and verified by sequencing through the Sequencing Core of Loyola University Medical Center. Successful expression of all proteins encoded by eukaryotic expression vectors included in vaccines, including hHSP70, mHSP70, and TRP-2, was confirmed by western blotting of total protein from transfected COS cells, followed by indirect alkaline phosphatase immunostaining.

Western blotting

COS cells were transfected with individual plasmids encoding human HSP70i, mouse HSp70A1A, and human TRP-2 using lipofectamine reagent (Invitrogen) according to manufacturer's instructions. Total proteins were isolated after 48 hours, and protein content was measured using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA), for equal loading at 12.5 μg for cellular proteins, or 5 μg of purified HSP60 or HSP70 per slot onto a 10% polyacrylamide minigel. After electrophoresis, separated proteins were transferred on to Immobilon-P membrane (Millipore, Billerica, MA). The blots were incubated with anti-HSP70 Ab (rabbit polyclonal Ab at 1:1,500 or mouse mAb SPA-810 at 1:2,000; Assay Designs, Ann Arbor, MI), anti-TRP-2 Ab (goat polyclonal Ab at 1:1,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA), or incubated with pooled sera from five vaccinated mice as the primary Abs (1:100 each). After washing, blots were reacted with alkaline phosphatase-conjugated secondary Abs (goat anti-rabbit, rabbit anti-goat, or goat anti-mouse at 1:1,000; Santa Cruz Biotechnology Inc.). Blots were developed with 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (Sigma-Aldrich, St Louis, MO) as the substrate.

Bullet preparation and gene gun vaccination

To prepare bullets, endotoxin-free plasmid DNA in desired combinations was precipitated onto spermidine-coated gold beads (Fluka Biochemika, Buchs, Switzerland and Sigma-Aldrich) in the presence of 200mM $CaCl_2$ (Sigma, St Louis, MO) and 10 volumes of ethanol (Sigma). Washed beads were precipitated onto silicone tubing (Bio-Rad) in a BioRad Tubing Prep Station (Bio-Rad). Bullets were used within 10 days of preparation.

Two strains of mice (C57BL/6J from Jackson Labs, Bar Harbor, ME, and 129S7 from Taconic, Hudson, NY) were included in experiments. Group sizes were 5 or 10 mice per group as indicated in the figure legends. All experiments were approved by Loyola University Medical Center's Institutional Animal Care and Use Committee. Mice were

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prepared for weekly gene gun vaccination by biweekly ventral hair removal with Nair (Church and Dwight Co., Princeton, NJ) and injections of ketamine (ketaject, IVX Animal Health, St Joseph, MO) at 95 mgkg^{-1} and xylazine (IVX Animal Health) at 5 mg kg^{-1}, and vaccinated with 4.8 μg of DNA for four consecutive weeks. By gene gun vaccination using the Helios Gene Gun System (Bio-Rad), gold particles coated with DNA of interest are released from silicon tubing cartridges under helium pressure at maximum 300 p.s.i. (pound per square inch), which allows for DNA to directly enter the skin and nestle inside relevant cell types such as DC, where the DNA can be expressed before and after migration to draining lymph nodes to induce an immune response to antigens encoded by the vaccine.

Evaluating depigmentation

Depigmentation was evaluated by scanning front and back of the mice with a flatbed scanner under anesthesia, and subsequent image analysis using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA). The percentage of depigmentation was calculated from the largest evaluable area as the percentage of pixels among >150,000 evaluated with a luminosity above the cutoff level set to include 95% of pixels for untreated mice. Statistical analysis of data was performed by comparing relevant groups by Student's *t*-test using Excel software.

Immunohistology

At euthanasia, biopsies of mouse skin were embedded in OCT compound (Sakura Finetek USA, Torrance, CA) and snap-frozen in liquid nitrogen. A measure of 8 μm cryostat sections were fixed in cold acetone and stored at −20 °C until use. Sections were stained with polyclonal Abs D18 to TRP-2 (goat polyclonal; Santa Cruz Biotechnology Inc.), C20 to S100 (Santa Cruz Biotechnology Inc.), or biotinylated 1452C11 to CD3ε (Armenian Hamster monoclonal; Pharmingen, San Diego, CA) in an indirect immunoperoxidase staining procedure essentially, as described previously (Le Poole *et al.*, 1993a, b). Qualitative evaluation of sections from each group was performed by two independent investigators.

In vivo cytotoxicity assessment

For *in vivo* cytotoxicity, fresh spleen cells were harvested from C57BL/6J mice, and separate batches were pulsed with relevant TRP-2-derived, K(b)-restricted peptide 180–188 SVYDFFVWL (a kind gift from Dr Ross Kedl, UCHSC (University of Colorado Health Sciences Center), Denver, CO) and irrelevant HPV16E7-derived, D(b)-restricted peptide 49–57 RAHYNIVTF (a kind gift from Dr Martin Kast, USC, Los Angeles, CA), then pulsed with 8 and 0.5 μM carboxyl fluorescein succimidyl ester (CFSE) (Invitrogen), respectively. Labeled cells were periorbitally injected into mice of differentially vaccinated mouse groups 5 days after a booster gene gun vaccination. Eighteen hours after injection, mice were killed, and samples representing 5,000 recovered irrelevantly pulsed spleen cells were scanned using FACScanto equipment (BD Biosciences, Sparks, MD). The FACScanto is a benchtop flow cytometer that contains a 15mV argon-ion laser and a red diode laser, and has the capability for detection of six fluorescent detection channels plus right and forward angle scatter.

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Abbreviations

Ab	antibody
DC	dendritic cell
HSP	heat shock protein
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
4-TBP	4-tertiary butyl phenol
TRP	tyrosinase-related protein

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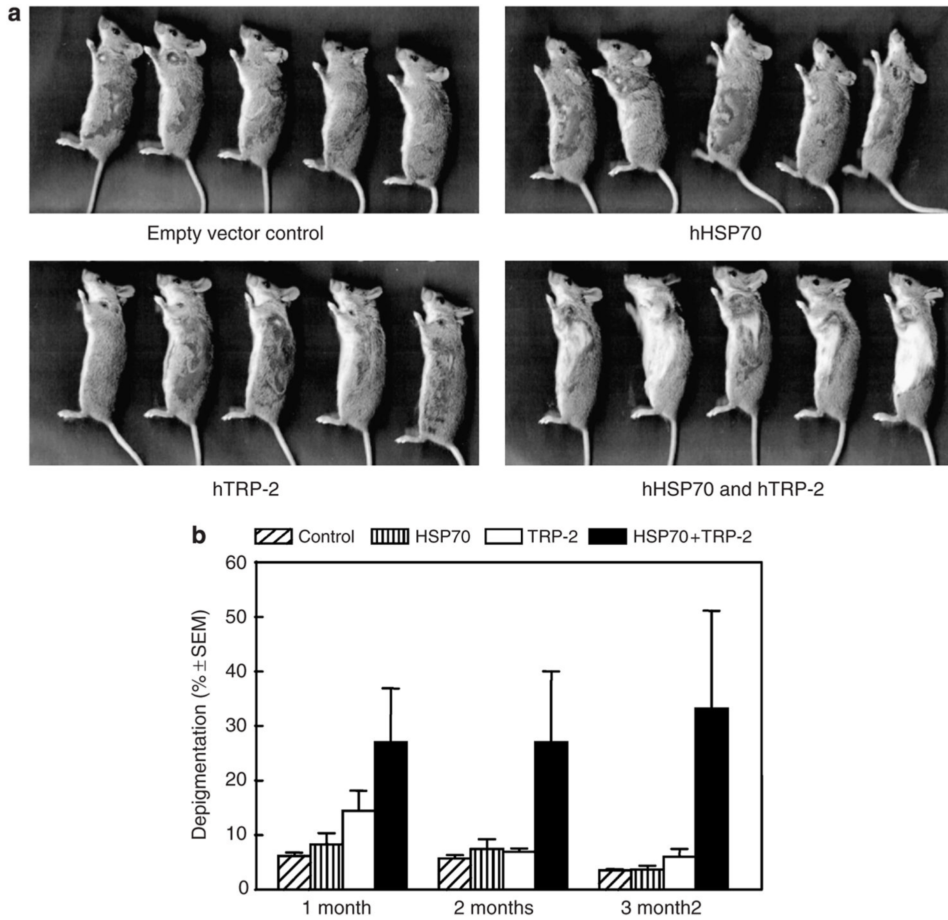

Figure 1. hHSP70 accelerates depigmentation in 2 different mouse strains

(**a**) HSP70 induces depigmentation in 129S7 agouti mice. Ten mice per group received 4 weekly vaccinations to the flank to avoid the off-white/yellow ventral region of this mouse strain, where depigmentation is difficult to appreciate. Vaccinations consisted of 4.8 μg DNA delivered by biolistic gene gun, containing combinations of expression plasmids encoding hTRP-2 and/or hHSP70, or empty vector control DNA. Images were prepared 6 weeks after the final vaccination. (**b**) Depigmentation is accelerated in response to HSP70. C57BL/6 mice (10 per group) were gene gun vaccinated weekly with 4.8 μg plasmid DNA encoding hTRP-2 and/or hHSP70, or empty vector control plasmid by biolistic gene gun vaccination to evaluate depigmentation in response to HSP70 in a mouse strain different from 129S7 shown in panel (a). Ventral depigmentation was quantified starting 1 month after vaccination.

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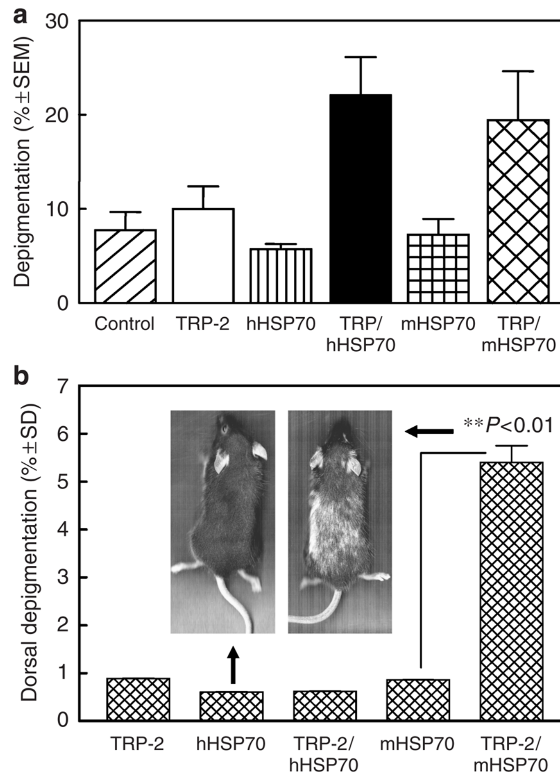

Figure 2. Progressive depigmentation induced by mHSP70 involves areas not exposed to stress

(**a**) mHSP70 similarly contributes to accelerated depigmentation in mice. Groups of 10 C57BL/6 mice were gene gun vaccinated weekly with 4.8 µg DNA consisting of (combinations of) expression plasmids encoding TRP-2 and/or HSP70, or no inset. HSP70 was either of human or mouse origin. Ventral depigmentation as shown was measured 3 weeks after the final vaccination. The data demonstrate that accelerated depigmentation by hHSP70 cannot be ascribed to its xenogeneity. (**b**) Dorsal depigmentation in C57BL/6 mice vaccinated in the ventral region. Mice were vaccinated as indicated in panel (a). Dorsal scans taken 8 weeks after the final vaccination are shown. Insets: example mice vaccinated weekly for 4 weeks with 2.4 µg DNA encoding human HSP70 and 2.4 µg empty control vector (left) or 2.4 µg mouse hsp70-encoding DNA combined with 2.4 µg human TRP-2 encoding DNA (right), showing dorsal depigmentation only in the latter group.

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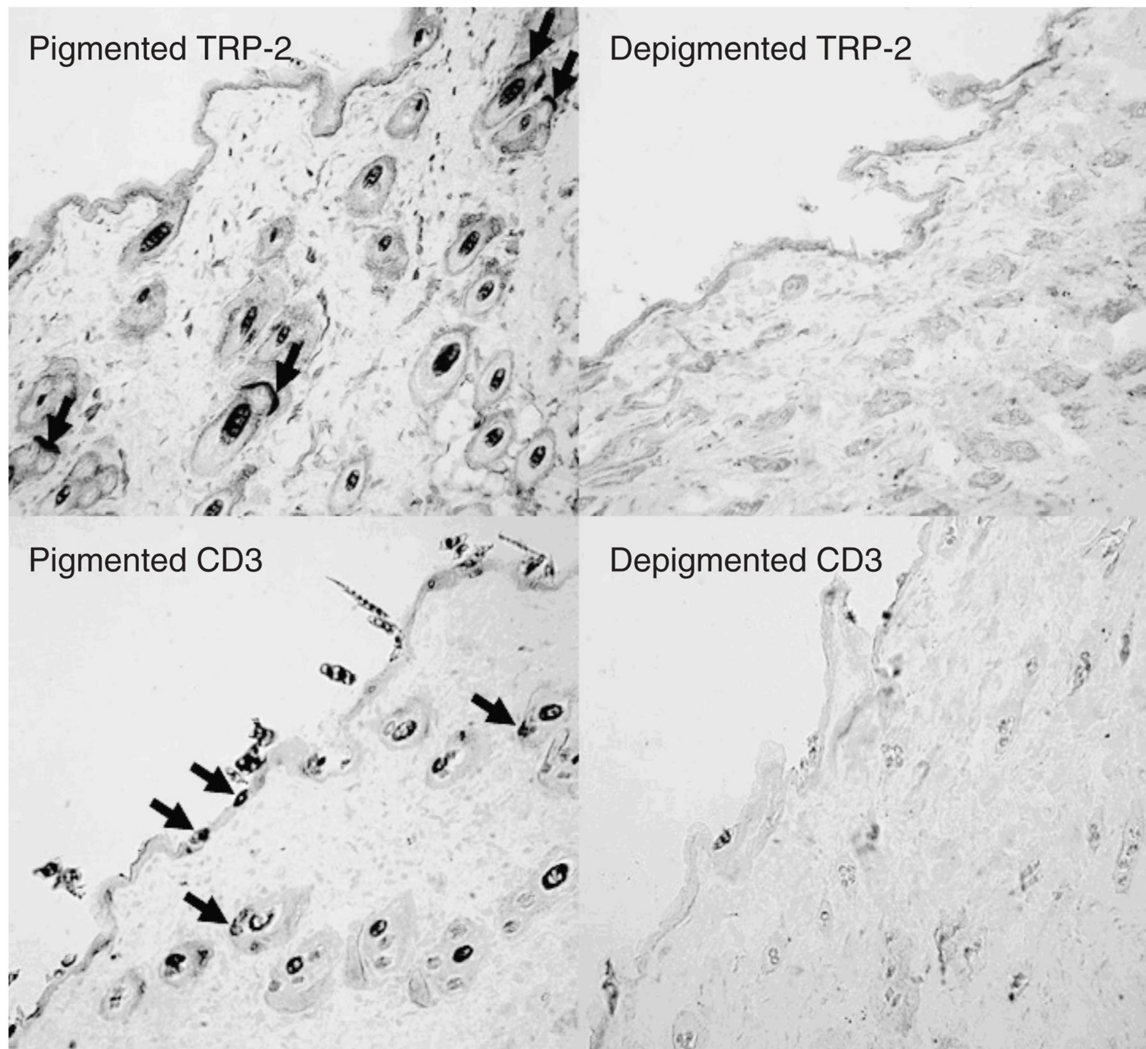

Figure 3. Gradual depigmentation of the pelage is associated with loss of melanocytes and T cells from the skin

TRP-2 and CD3ε immunostaining shown in cryosections from representative biopsy of ventral skin vaccinated with control plasmid (TRP-2 pigmented, CD3 pigmented) and a combination of TRP-2 and hHSP70-encoding plasmids (TRP-2 depigmented, CD3 depigmented). Arrows indicate melanocytes as detected by Abs to TRP-2, or T cells as detected by Abs to CD3ε, respectively.

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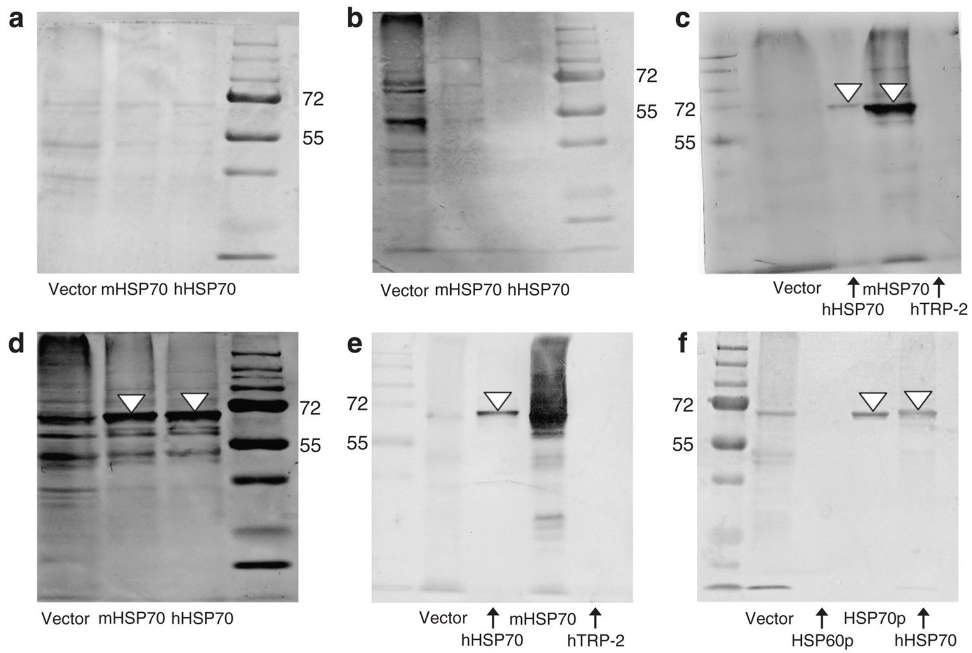

Figure 4. Vaccination with HSP70 encoding vectors induces a humoral response to the protein
Sera from five mice were pooled and reacted with western blots of proteins from transfected COS cells or purified stress proteins as indicated. Sera used were from mice vaccinated with plasmids encoding (**a**) TRP-2, (**b**) empty vector control, (**c**) hHSP70, (**d**) hHSP70 and TRP-2, (**e**) mHSP70 and TRP-2, and (**f**) hHSP70 and TRP-2. Prominent reactivity to a 70 kDa protein was observed only in sera from mice vaccinated in (combinations of) plasmids encoding human or mouse-derived HSP70. Such reactivity was not observed in mice vaccinated with TRP-2-encoding plasmid or control plasmid alone; no reactivity to TRP-2 was observed in any of the pooled sera. In blot (**f**), the 70 kDa detected protein was positively identified as HSP70. As a negative control, no reactivity to HSP60 was observed. The band of interest is highlighted by an arrowhead. Note that COS cells express some HSP70 regardless of transfection. HSP60P and HSP70P: purified proteins and hHSP70, mHSP70 and hTRP-2: cos cells transfected with plasmids encoding named proteins.

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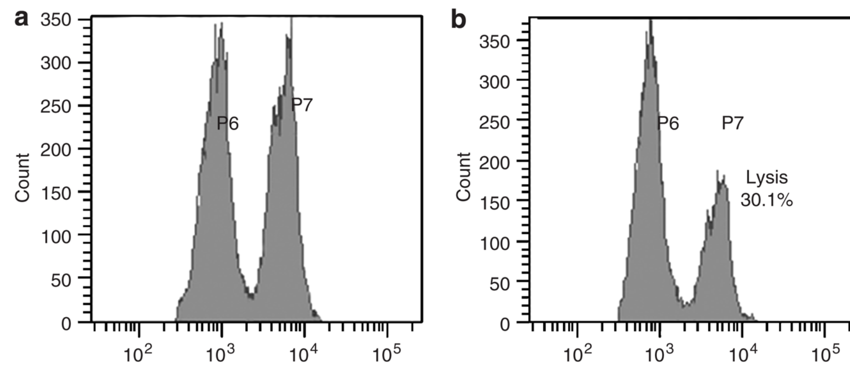

Figure 5. Depigmentation is associated with a cytotoxic response to TRP-2-expressing melanocytes

For *in vivo* cytotoxicity assays, all mice were challenged with an equal number of spleen cells loaded with an irrelevant peptide plus 1 μM CFSE (left peak P6, $CFSE^{low}$), or a TRP-2-derived peptide and 8 μM CFSE (right peak P7, $CFSE^{high}$). Spleens from challenged mice are harvested after 18 hours and analyzed by FACS. Cytotoxicity was calculated from the relative area underlying either peak, with loss of cells from the $CFSE^{high}$ peak ascribed to cytotoxic T cells targeting $TRP\text{-}2_{180–188}$. Histograms are shown for representative mice that were vaccinated with (**a**) hHSP70 alone or (**b**) hHSP70 and hTRP-2, 5 days before the *in vivo* cytotoxicity challenge.