

LOYOLA UNIVERSITY CHICAGO

SEPARATING AUTOIMMUNE
AND ANTI-TUMOR RESPONSES WITH HSP70^{iQ435A}

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BY

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ABSTRACT

Vitiligo is described as an autoimmune-mediated skin disorder in which melanocytes are targeted and killed by cytotoxic lymphocytes, resulting in depigmentation of the skin. One cause of vitiligo progression is when stressed or damaged melanocytes release a 70kDa stress-induced heat shock protein (HSP70i) carrying melanocyte protein; this activates nearby dendritic cells toward an inflammatory phenotype which circulate through the lymph and prime a cytotoxic response against melanocyte antigen. The Le Poole lab previously identified a peptide within HSP70i responsible for activating this inflammatory phenotype in human DCs and developed HSP70i_{Q435A}. It was found that treatment with HSP70i_{Q435A} is able to prevent proliferation of the vitiligo-associated inflammatory DC phenotype, thus preventing accumulation of melanocyte antigen-specific T cells in the skin and reducing depigmentation in mice.

However, because treatment with HSP70i_{Q435A} inhibits this DC-mediated autoimmune response against melanocytes in vitiligo mouse models, there existed a valid concern that treatment with this modified heat shock protein may also inhibit natural anti-tumor responses against melanocyte-derived melanoma tumor cells. Therefore, the first hypothesis of this project is that HSP70i_{Q435A} prevents migration of melanocyte-antigen reactive T cells to the skin in a way that is mechanistically different from anti-tumor responses to melanoma and therefore would not have a negative effect on anti-

tumor responses. Using data gathered from testing this first hypothesis, a second hypothesis was established, proposing that treatment with HSP70i_{Q435A} confers anti-melanoma resistance through antibody-dependent cell-mediated cytotoxicity.

To test the first hypothesis, BL/6 mice were treated with DNA-vaccines encoding WT HSP70i, HSP70i_{Q435A}, or were not vaccinated for 5 weeks and then tumor-challenged with B16 mouse melanoma. Results from several of these vaccination/tumor-challenge mouse experiments displayed that treatment with both WT HSP70i and HSP70i_{Q435A} inhibit tumor growth in the subsequent tumor challenge when compared to the non-vaccinated mice. CD3 T cell migration to the melanoma was not negatively affected in the treated mice. Surface staining of B16 melanoma cells also revealed surface expression of HSP70i, consistent with past observations of tumor cell overexpression and surface expression of heat shock proteins. These data suggest that anti-tumor responses following treatment with HSP70i_{Q435A} are not inhibited but actually strengthened, and this anti-tumor response may be related to the overexpression of HSP70i common to tumor cells.

To test the second hypothesis, B-Cells were adoptively transferred from HSP70i DNA-vaccinated BL/6 mice into naïve BL/6 that then received a tumor challenge. Results displayed that mice receiving the adoptively transferred B cells had decreased tumor growth rates and final tumor mass, when compared to tumor-challenged mice that did not receive adoptively transferred cells. Furthermore, serum taken from vaccinated animals (mouse and swine) show increased levels of anti-HSP70i antibody. Taken together, these data suggest that HSP70i_{Q435A} polarizes dendritic cells to support

antibody-mediated responses to surface HSP70i expressed by tumor cells while preventing migration of melanocyte-antigen reactive T cells to the skin.

CHAPTER 1

INTRODUCTION

Vitiligo Background

Vitiligo is described as an autoimmune-mediated skin disorder in which melanocytes are killed, resulting in depigmentation of the skin. During vitiligo, melanocytes are destroyed by melanocyte-reactive T cells, as well as other immune or non-immune components (1). Expanding vitiligo lesions are consistently infiltrated with T cells in the areas lining the lesional borders; these infiltrating T cells are composed primarily of CD8+ cytotoxic T cells reactive with melanocyte-specific antigens (2). Moreover, increased numbers of inflammatory CD11c+ dendritic cells have been observed in the lesional skin of vitiligo patients, which are thought to lend to the increased auto-immune response leading to vitiligo (3).

However, problems arise in the development of effective therapeutics for the disease because re-establishing pigmentation in patients with active disease would likely be problematic without first halting progression of the disease; in this sense, understanding the cellular mechanisms behind depigmentation in vitiligo disease became a key focus of vitiligo studies.

Previous research into the mechanisms behind vitiligo progression has demonstrated a link between stress-induced heat shock proteins as players involved in

the autoimmune responses responsible for vitiligo (5). Primary among these immune-activating stress proteins are HSPs 60, 70, and 90 (5). Certain HSPs assist in normal cellular functions such as protein-misfold repair; others are synthesized only under stress (12). Of interest among these stress-inducible HSPs is an inducible form of Heat Shock Protein 70 (HSP70i, also known as HSP72) which is known to be secreted from live cells through an active-release mechanism (9). Under standard physiological conditions, the constitutive isoform of HSP70 (HSC70) is expressed within the cell offering cytoprotection to the cell through assisting in chaperoning misfolded proteins. However, through cell death or active secretion, an inducible isoform of HSP70 (HSP70i) can be released into the extracellular environment where it is then able to interact with dendritic cells to enhance the uptake of antigens (7). This implies that an auto-immune response to HSP70i, and any proteins it may be chaperoning, can occur even in the absence of cell death.

HSP70 and Vitiligo

In vitro studies have associated HSP70 as a factor in the formation of the vitiligo disease state: melanocytes were shown to overexpress HSP70 in response to the bleaching agent 4-tertiary butyl phenol (4-TBP), a compound used in rubber manufacture and the photoindustry, which is selectively cytotoxic to melanocytes within the skin and induces depigmentation in vitiligo patients sensitive to these agents (3). Increased amounts of HSP70 were detectable in the supernatant of vitiligo melanocytes compared to control melanocytes (3). Studies comparing the lesional and non-lesional skin of three

vitiligo patients show consistent differential expression of HSP70 as observed by immunohistological staining, further implying that HSP70 plays a role for in autoimmune-induced depigmentation of melanocytes (8).

HSP70i_{Q435A}

Previously, the Le Poole lab identified a peptide within the stress-inducible HSP70i responsible for activating human dendritic cells and developed HSP70i_{Q435A}, a mutant DNA isoform with a single amino acid modification. Located on the outer cusp of the substrate binding domain in 3D models of the protein, just outside the substrate binding groove, this change in amino acid 435 affects activation of DCs following binding with the stress protein. It was found that vaccination with this mutant form of HSP70 (*HSP70i_{Q435A}*) is able to prevent proliferation of the vitiligo-associated inflammatory dendritic cell phenotype, thus preventing accumulation of melanocyte antigen-specific T cells in the skin and reducing depigmentation in mice (4). These findings suggested that the mutant form HSP70i is not only an inactive variant but also binds DCs and alters their function in such a way that interferes with later T cell activation. The implications of this study are that HSP70i_{Q435A} DNA delivery may be used for the treatment of vitiligo (4).

Melanoma Background

While vitiligo deals with melanocyte death, at the other end of the spectrum we have the uncontrollable proliferation of melanocytes in the form of melanoma skin cancer. Skin cancer, the current most common malignancy across the world, is often classified as either non-melanoma skin cancer (NMSC) or melanoma skin cancer (14).

NMSC, which is rarely lethal, accounts for the majority of skin cancer cases, with the most common types being basal cell cancer and squamous cell cancer. Malignant melanoma skin cancer, while accounting for only ~4% of all skin cancers, is the major cause of death from skin cancer (15). Melanoma is derived from pigment-producing melanocytes which, within in the skin, are found at the basal level of the epidermis (14). Immune suppression in the skin, induction of uncontrolled melanocyte cell division, or DNA damage of growth regulatory genes caused by sources such as UV radiation or reactive oxygen species (16) all contribute to the development and progression of melanoma. While non-metastatic melanoma can be treated with surgical removal if detected early, later stage metastatic malignant melanoma is extremely difficult to treat with existing cancer therapies and often has a poor prognosis, with a median survival rate of 6 months and a 5-year survival rate of less than 5% (15). In 2012, the American Cancer Society reported 1.6 million new cases of skin cancer, with about 76,000 of those cases diagnosed as melanoma; of the 12,190 deaths caused by skin cancer in 2012, almost 9,200 were caused by melanoma (14). For a malignant melanoma cell to be successful it must persist in incredibly high-stress environments; under these difficult conditions, melanoma cells often upregulate stress proteins as a means of promoting protein homeostasis and cellular viability (13).

Link between HSP70i, Vitiligo, and Melanoma

The overexpression of HSP70i in these tumor cells is often associated with secretion and membrane-expression of stress proteins with evidence to suggest that HSP70i is one of these stress proteins is that is overexpressed and expressed on the

membrane of primary and metastatic melanoma cells but not normal cells; as such, HSP70i may be a viable target for anti-melanoma therapeutics (10). This information becomes even more exciting when you consider that vitiligo is known to be an unavoidable side-effect of certain melanoma therapeutics which target melanoma-antigen, as there is often crossover between antigen expressed on melanoma cells and antigen expressed on melanocytes, resulting in an immune response toward the melanoma cell that collaterally attacks healthy melanocytes. With this in mind, there is a concern that treatment for vitiligo with this mutant HSP70i may alter the autoimmune response in such a way that the immune cells directly affected by HSP70i interaction (DCs, T-Cells) would not as effectively target melanoma tumor.

HYPOTHESIS

HSP70i_{Q435A} polarizes dendritic cells to support B cell responses to surface HSP70i expressed by tumor cells while preventing migration of melanocyte-antigen reactive T cells to the skin.

The following specific aims were established to test the hypothesis.

SPECIFIC AIMS

Aim I: Measure melanoma and vitiligo development in response to HSP70i_{Q435A} vaccination.

These experiments will help shed light on the consequences of HSP70i_{Q435A} vaccination with regards to anti-melanoma immunity, and whether or not tumor containment is affected by this change in auto-immunity.

Sub-Aim I: Determine the effect of HSP70i_{Q435A} vaccination on tumor growth without eliciting depigmentation.

Vaccinate WT C57BL/6 mice with HSP70i_{Q435A} and challenge with B16-F10 melanoma cells, following tumor growth and depigmentation over time.

Sub-Aim II: Determine if HSP70i_{Q435A} vaccination is able to inhibit depigmentation in vitiligo-prone mice while inhibiting tumor growth.

Vaccinate PMEL-1 mice and challenge with B16 melanoma cells, following tumor growth and depigmentation over time.

Sub-Aim III: Begin to examine the effects of HSP70i_{Q435A} vaccination in vitiligo-prone swine to address the bioavailability, safety, and efficacy of a DNA vaccine designed to treat vitiligo.

Treat swine vitiligo lesions with DNA vaccine and assess changes in lesional size over time.

Aim II: Establish the cell(s) responsible for tumor containment and depigmentation.

These experiments will help us understand the mechanism by which treatments with the DNA-vaccines encoding HSP70i_{Q435A} and WT HSP70i are able to promote anti-melanoma responses in mice.

Sub-Aim I: Examine humoral responses to HSP70i after vaccination with the HSP70i_{Q435A} clinical vector.

Immune monitoring of available serum and relevant tissues from animals after HSP70i_{Q435A} vaccination or adoptive transfer.

Sub-Aim II: Determine which immune cells, if any, are involved in tumor containment as a result of HSP70i_{Q435A} vaccination.

Vaccinate WT C57BL/6 mice with HSP70i_{Q435A} and adoptively transfer immune cells from the vaccinated mice to B16-challenged SCID/beige immune-deficient recipient mice.

CHAPTER 2

AIM I: Measure melanoma and vitiligo development in response to HSP70i_{Q435A} vaccination.

SUB-AIM I: Determine the effect of HSP70i_{Q435A} vaccination on tumor growth without eliciting depigmentation.

EXPERIMENTAL APPROACH

To prepare the bullets for gene gun vaccination, 100 µg of endotoxin-free plasmid DNA of both WT HSP70i and HSP70i_{Q435A} (prepared in pUMVC3 DNA vector, designed by the University of Michigan's Vector Core) was precipitated onto spermidine-coated gold micro-particles in the presence of 200mM CaCl₂ and washed with 6 volumes of ethanol. Washed beads were precipitated onto silicone tubing in a BioRad Tubing Prep Station and prep. The DNA-coated tubing was cut into sections that fit the Helios Gene Gun (BioRad), with about 1.2 µg of DNA per bullet. Bullets were used within 2 weeks of preparation.

WT C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were used in this experiment. The mice were separated into 3 vaccination groups: control, WT HSP70i, and HSP70i_{Q435A}. *For each group, n=6.* Mice were prepared for gene gun vaccination by biweekly ventral hair removal with Nair. The mice were anesthetized using 5% isoflurane and vaccinated with four bullets each (4.8 µg of DNA) for five consecutive weeks (one vaccination every

6 days). During gene gun vaccination with the Helios Gene Gun, gold micro-particles coated with DNA are released from silicon tubing cartridges under helium pressure at a maximum of 300 p.s.i., which allows for the DNA to directly enter the skin and nestle inside relevant cell types such as DC, where the DNA is mechanically transfected and expressed.

One week after the final vaccination, the mice were sub-cutaneously challenged with 10^5 cells of the B16 mouse melanoma cell line; the tumors were injected near the sites of vaccination, once in the left flank and once in the right flank of each mouse. Tumor progression was followed over the next several weeks using a pair of calipers to determine tumor volume in each mouse. Two weeks after the first sign of a palpable tumor (which took 8 days to emerge post-injection) the mice were sacrificed; their final tumor volumes were measured and the tumors were harvested to be weighed for mass and cryopreservation.

RESULTS

The experiment outlined in the experimental approach is a repeat of a vaccination experiment performed during my initial rotation in the lab; during this initial vaccination the mice were vaccinated with WT HSP70 in TOPO 3.1 GFP vector and HSP70_{iQ435A} in TOPO 3.1 GFP vector (rather than pUMVC3 vector, as described in the previous experimental approach section). It was repeated again using the pUMVC3 vector because the pUMVC3 vector is approved for clinical trials while the TOPO 3.1 vector is not.

In the pUMVC3 vector vaccinated mice, tumor progression was followed after the first sign of tumor growth (Day 0). When compared to the unvaccinated mice, the vaccinated mice displayed a longer onset of tumor growth (by about 3 days), an average decreased tumor growth rate as a measure of volume, and smaller tumors on average as a measure of volume (66% decrease in final tumor size for the HSP70i_{Q435A} vaccinated mice, 62% decrease in final tumor size for the WT HSP70i vaccinated mice).

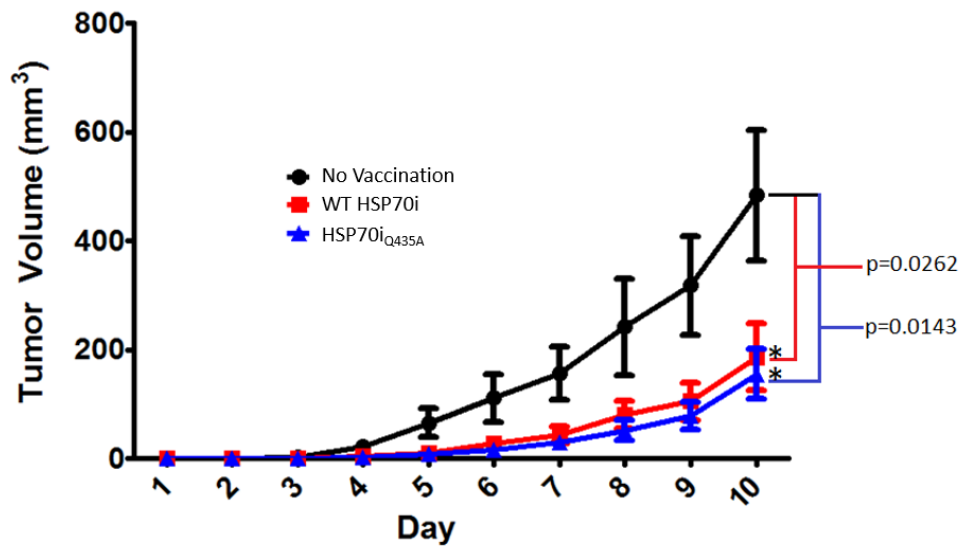


Figure 1: Tumor Growth is slowed in mice vaccinated with pUMVC3 vector DNA encoding WT HSP70i and HSP70i_{Q435A}.

As seen in Figure 1, the difference in combined tumor volume by the end of the experiment displayed significance for both vaccination groups ($p=0.0143$ between Control and HSP70i_{Q435A}, $p=0.0262$ between Control and WT HSP70i). Final tumor volumes

were analyzed for significance using an unpaired, two-tailed T-Test with a confidence interval of 95%. (n = 6 for each group).

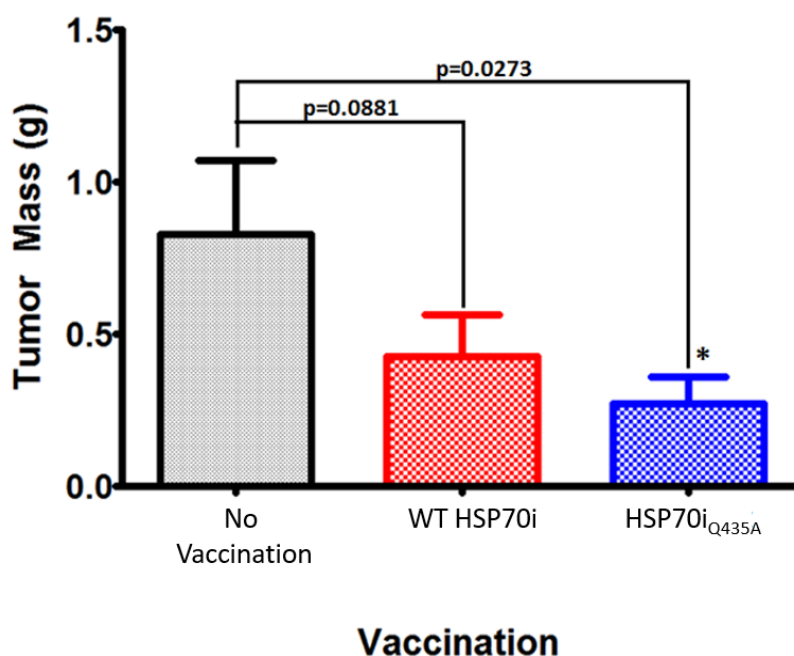


Figure 2: Mice vaccinated with pUMVC3 vector DNA encoding HSP70i WT and HSP70i_{Q435A} show reduction in average tumor mass.

After sacrifice of the animals, the tumors were harvested, weighed, and the average tumor masses per vaccination group were compared. As seen in Figure 2, a student's T-Test revealed a significant 70% decrease in average tumor mass of the HSP70i_{Q435A} vaccinated animals when compared to the control animals (p=0.0273), while the WT HSP70i vaccinated animals trended toward a smaller average tumor mass than the control animals but did not reach significance (p=0.0881).

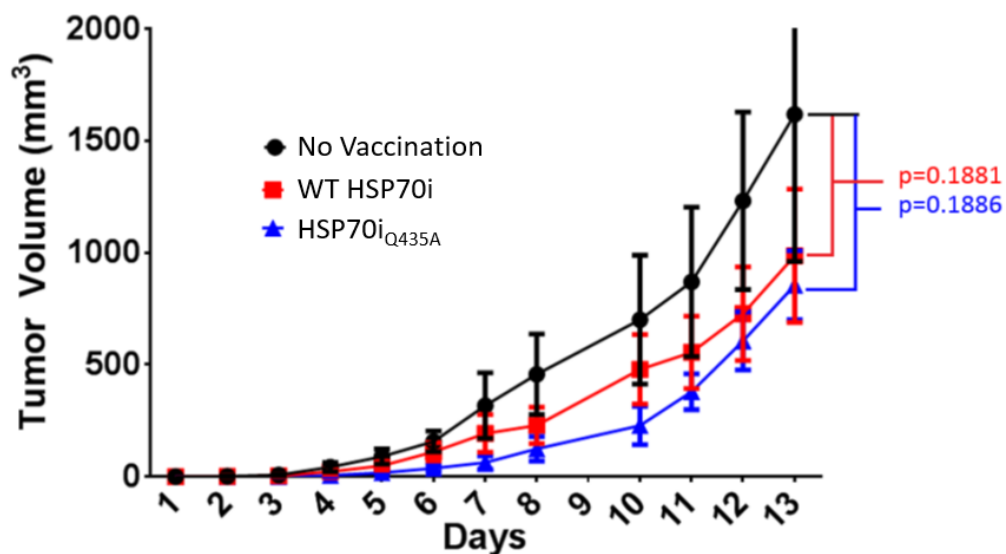


Figure 3: Trend toward decreased melanoma tumor growth seen in mice vaccinated with TOPO vector DNA encoding WT HSP70i and HSP70i_{Q435A}.

For reference, tumor growth data from the original TOPO vector vaccinated mice are included as follows. The vaccinated mice represented in Figure 3 displayed a longer onset of tumor growth, an average decreased tumor growth rate as a measure of volume, and smaller tumors on average as a measure of volume. The vaccinated mice trended towards smaller average tumor volumes when compared with the average tumor volume of unvaccinated mice ($p=0.1886$ between Control and HSP70i_{Q435A}, $p=0.1881$ between Control and WT HSP70i) ($n = 5$ for each group).

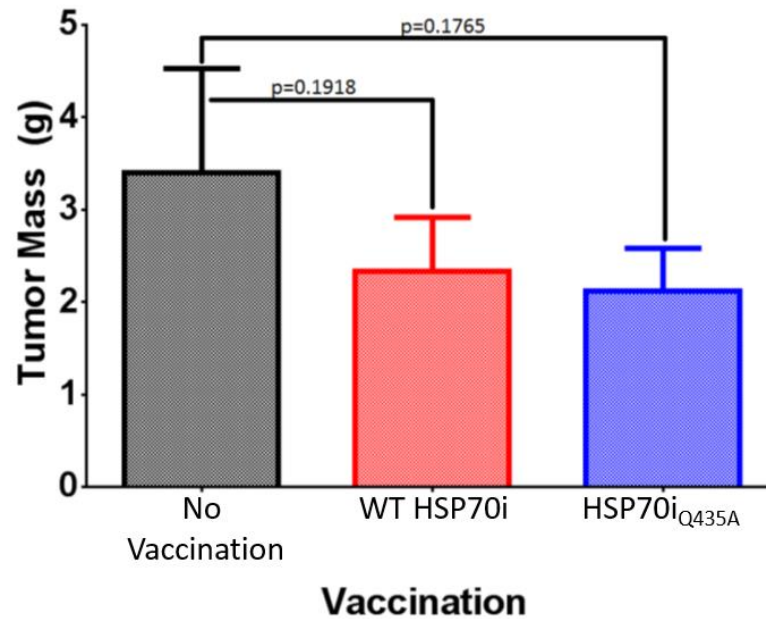


Figure 4: Mice vaccinated with both forms of HSP70i in TOPO vector show a trend in reduction of tumor mass.

As seen in Figure 4, the difference in both the average tumor mass of the HSP70i_{Q435A} vaccinated animals and the average tumor mass of the WT HSP70i vaccinated animals trended toward a smaller tumor mass than the tumors of the control animals, but did not reach significance ($p=0.1765$ for the HSP70i_{Q435A} vaccinated animals, $p=0.1918$ for the WT HSP70i vaccinated animals). ($n = 5$ for each group)

Together, these data showing significantly decreased tumor growth rates and final tumor mass in the vaccinated mice indicate that anti-melanoma responses are not inhibited, but strengthened by vaccination with both forms of HSP70i.

SUB-AIM II: Determine if HSP70i_{Q435A} vaccination is able to inhibit depigmentation in vitiligo-prone mice while inhibiting tumor growth.

EXPERIMENTAL APPROACH

The mice used in this experiment are transgenic PMEL-1 mice, which carry T cells with a rearranged T cell receptor specific for the mouse homologue (pmel-17) of human melanocyte protein gp100 and naturally develop vitiligo (11). Prior to vaccination the mice were imaged (dorsally and ventrally) using a photo-scanner to measure pre-vaccination pigmentation. Gene Gun bullet preparation and Gene Gun vaccinations were carried out as described in **Aim I: Sub-Aim I**. Mice were DNA-vaccinated with either 2.4µg of WT HSP70i in pUMVC3 plasmid DNA, or 2.4ug of HSP70i_{Q435A} in pUMVC3 plasmid DNA. Due to time constraints, each mouse was also DNA-vaccinated with 2.4ug of hGP100 in TOPO plasmid DNA; this was done to facilitate depigmentation. The mice received 5 vaccinations every 6 days, followed by 2 booster vaccinations 4 days apart. Over the course of the vaccinations, the mice were imaged using the photo-scanner immediately prior to their biweekly hair-removals.

One week after the final vaccination, the mice were sub-cutaneously tumor challenged with 10⁵ B16 mouse melanoma cells as described in **Aim I: Sub-Aim I**. Tumor progression was followed as a measure of volume while the mice were alive, and pigmentation was measured just before sacrifice. Tumors were harvested from the mice upon sacrifice and weighed for mass and cryopreservation. Statistical analysis was conducted on average final tumor volumes as well as average tumor masses to determine

the effect of HSP70i/ HSP70i_{Q435A} on tumor containment. Depigmentation/Repigmentation was also analyzed using scans taken before, during, and after treatment.

RESULTS

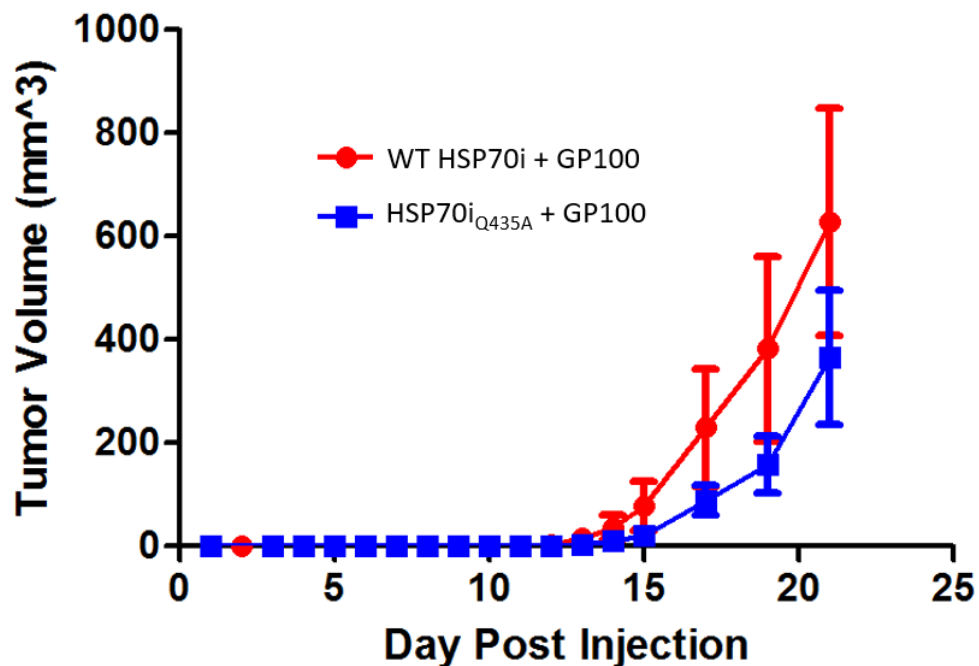


Figure 5: Average tumor growth rates from PMEL-1 mice following DNA-vaccinations and tumor challenge.

Tumor growth was measured after the first sign of tumor growth in WT HSP70i vaccinated mice on Day 12, and Day 13 in HSP70i_{Q435A} vaccinated mice. As seen in Figure 5, the mice vaccinated with HSP70i_{Q435A} displayed a longer onset of tumor growth and smaller tumors on average as a measure of volume when compared to the mice vaccinated with WT HSP70i, though not significantly different (n = 10 for each group).

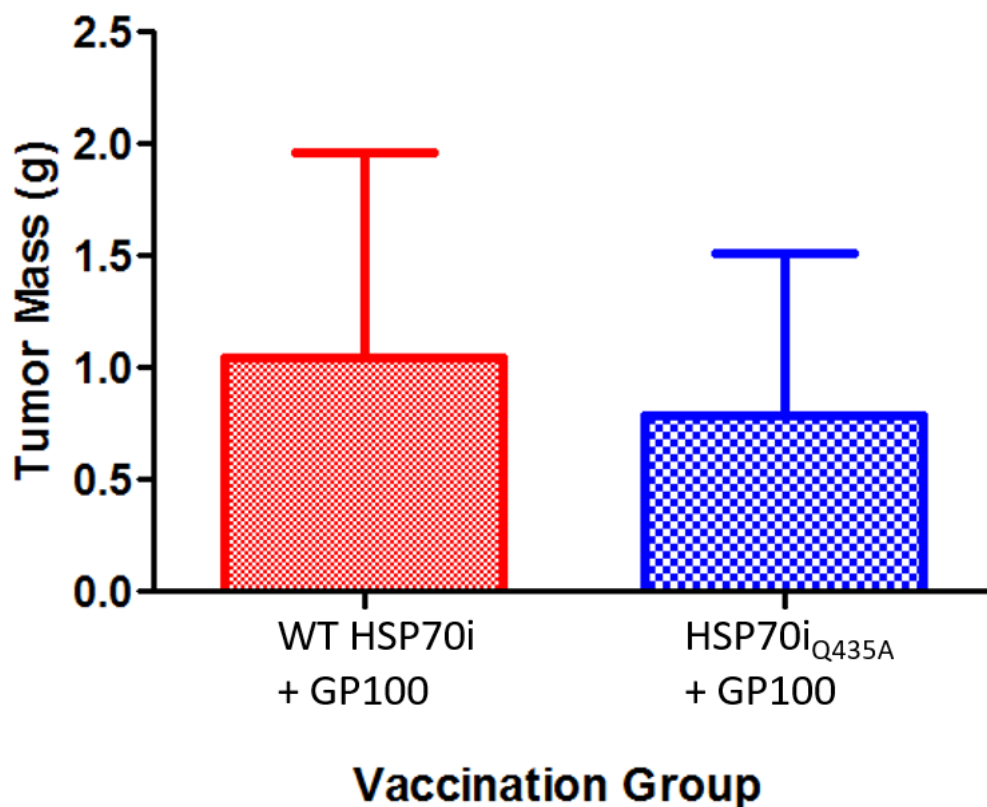


Figure 6: Final average tumor mass from PMEL-1 mice following DNA-vaccinations and tumor challenge.

As seen in Figure 6, the average final tumor mass of the HSP70i_{Q435A} vaccinated mice was 0.8g while the average final tumor mass of the WT HSP70i vaccinated mice was 1g; the average final tumor mass of the HSP70i_{Q435A} vaccinated mice was decreased, but not significantly different when compared to the WT HSP70i vaccinated mice. This data

further suggests that tumor containment is not impeded by DNA-vaccination with the mutant HSP70i_{Q435A}.

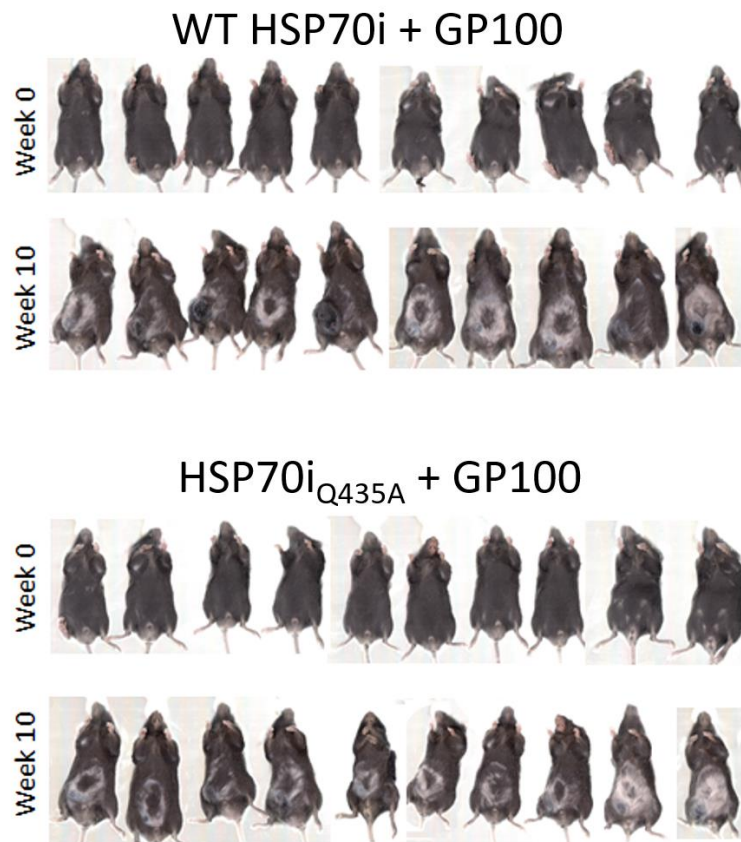


Figure 7: Images of PMEL-1 depigmentation over the course of the study.

Figure 7 shows images of mice taken immediately prior to treatment (Week 0) and immediately prior to sacrifice (Week 10). Areas of depigmentation can be seen along the abdomen where DNA vaccination took place (n = 10 for each group).

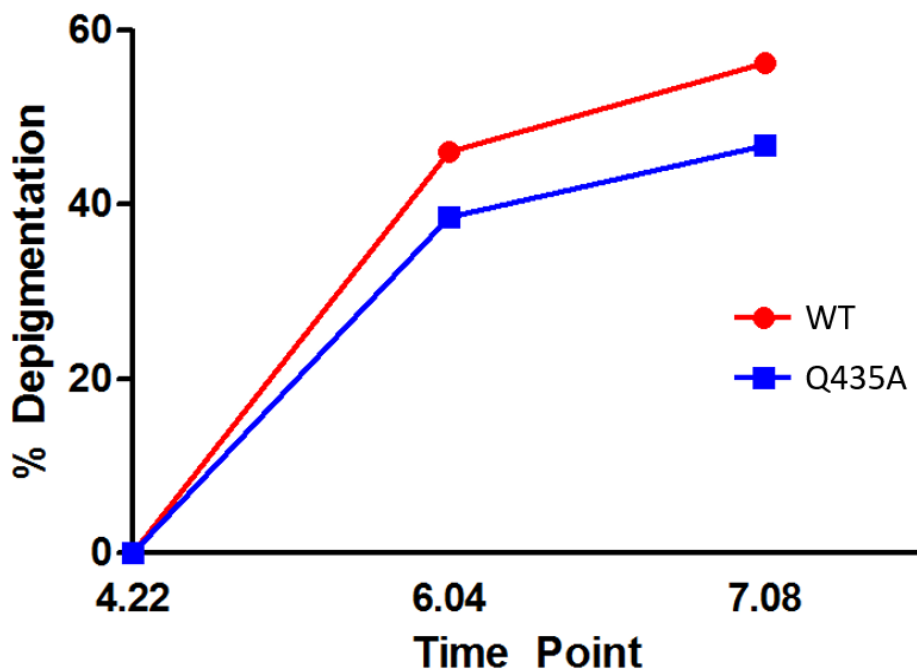


Figure 8: PMEL-1 average depigmentation over the course of the study might be trending toward decreased average depigmentation in HSP70i_{Q435A} vaccinated mice.

Depigmentation was analyzed as a measure of luminosity level change when compared to the individual animal's starting levels measured at 4.22. As seen in Figure 8 at the 6.04 time point, the WT HSP70i vaccinated mice display an average of 46% depigmentation, while the HSP70i_{Q435A} vaccinated mice display an average of 38% depigmentation, a 1.2 fold increase in WT over Q435A. At the 7.08 time point, the WT HSP70i vaccinated mice display an average of 55% depigmentation, while the HSP70i_{Q435A} vaccinated mice display an average 46% depigmentation, a 1.2 fold increase in WT over

Q435A. (n = 10 for each group). These depigmentation data indicate a small decrease in depigmentation in the HSP70i_{Q435A} treated mice, but no significant difference.

SUB-AIM III: Begin to examine the effects of HSP70i_{Q435A} vaccination in vitiligo-prone swine to address the bioavailability, safety, and efficacy of a DNA vaccine designed to treat vitiligo.

Bringing HSP70i_{Q435A} closer to the clinic, our lab plans to introduce and track the HSP70i_{Q435A} vaccine in swine with human-like skin and manipulate tumor growth and depigmentation in response to wild type and mutant HSP70i in Sinclair swine with spontaneous melanomas and vitiligo to address the microenvironmental signature of relevant sites.

EXPERIMENTAL APPROACH

Animals were treated weekly with 5 mg of the drug for a total of 4 weeks. A PharmaJet injector is being used to deliver 4 doses of 0 (control) or 1.25 mg/mL DNA in 0.5 mL each to the border of a single, preselected lesion. Animals were followed for 6 months from the first DNA vaccination. The primary objectives were to measure changes in lesional expansion (relative to the injection sites) by biweekly grid measurements and to follow drug distribution and local and systemic reactivity over time. Biweekly 50ml blood draws will be accompanied by monthly perilesional biopsies under local lidocaine anesthesia from treated and untreated perilesional skin before, during, and after treatment. For blood sampling and biopsies, pigs are secured and anesthetized with 2-4%

isoflurane using a mask. 4 mm biopsies were taken and skin were sutured with Vycril sutures or vetbond tissue adhesive, accompanied by topical application of triple antibiotic ointment as a preventive measure against infection. Animals were monitored until conscious before returning them to their cage, hourly thereafter until the animal appeared calm, and every next day until suture removal. Biopsies were snap-frozen on dry ice and stored at -80c until needed for cryosectioning and tissue staining. The pigs and lesional areas are followed for progressive depigmentation or repigmentation, similar to testing planned in human subjects, wherein grid measurements are made to quantify lesional area (including both treated and untreated lesions) at 2 week intervals before and after vaccination. At euthanasia, major tissues will be collected, screened, and evaluated for drug content. Remaining melanoma tumor mass will be measured. Tissues collected are stored frozen for further analysis of antibody titers by ELISA, and immunostaining to quantify immune infiltrates according to parameters previously established in mice [see Aim II: Sub-Aim II results].

RESULTS

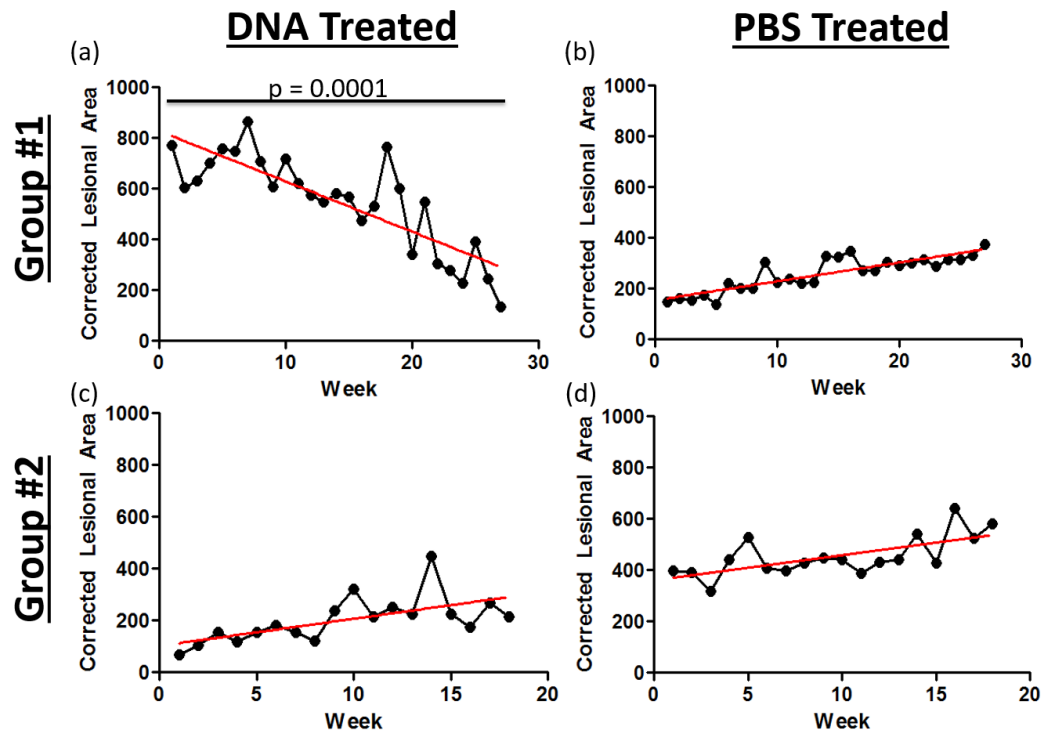


Figure 9: Treatment with DNA-vaccine encoding HSP70^{iQ435A} can induce repigmentation of lesional vitiligo in swine.

Lesional areas of interest were measured over the course of the study and changes in the vaccination-site lesion are represented in Figure 9. As seen in these data, the DNA treated swine in Group #1 displayed a very significant decrease ($p = 0.0001$) in vitiligo lesional area when comparing the starting time point with the final time point, decreasing in size 75% over the course of 27 weeks (9a). The Group #1 and group #2 PBS swine

showed increasing melanoma lesional size (9b, 9d). The Group #2 DNA treated swine displayed a 300% increase in lesional size (9c).

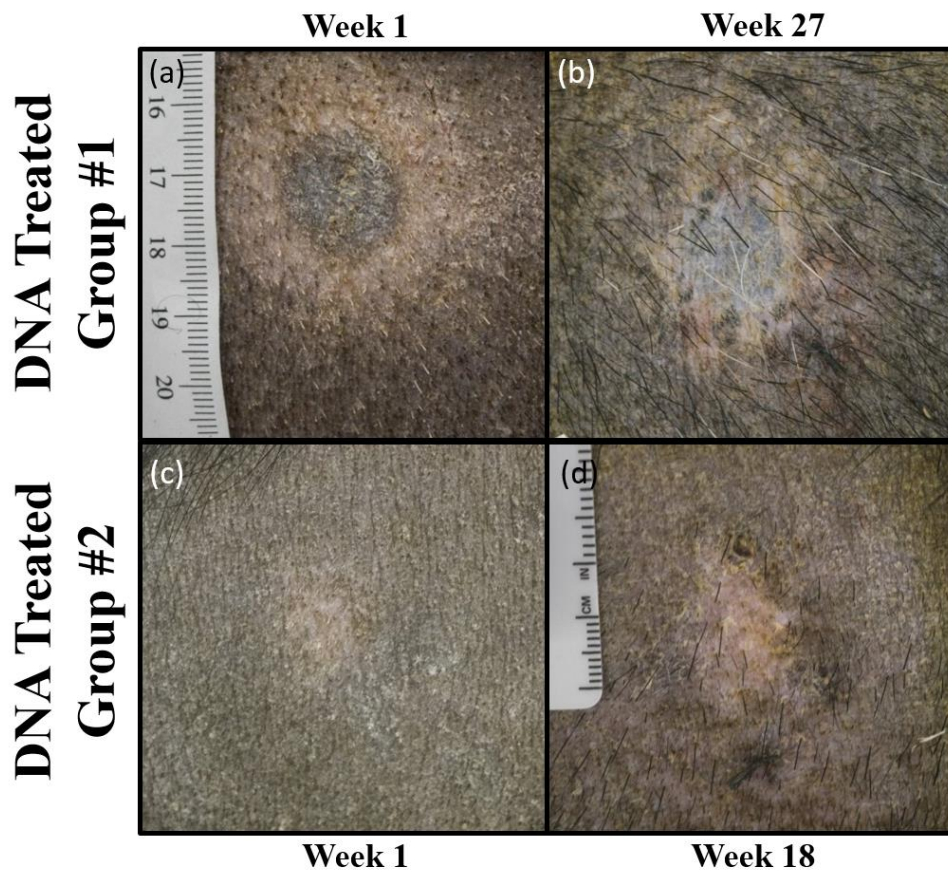


Figure 10: Treatment with DNA-vaccine encoding HSP70i_{Q435A} can induce repigmentation of lesional vitiligo in swine.

Figure 10 shows images of treated vitiligo lesions on both of the DNA treated swine; the images for the Group #1 swine were taken at Week 1 [10a] and Week 27 [10b] while the images for the Group #2 swine were taken at Week 1 [10c] and Week 18 [10d].

The Group #1 swine displayed a significant regression of its vitiligo lesion between Week 1 [10a] and Week 27 [10b], regressing about 78% from its original size. The Group #2 DNA treated swine displayed a 300% increase in lesional size from Week 1 [10c] to Week 18 [10d]. These data from the DNA vaccinated swine in Group #1 suggest a positive response to the drug in terms of vitiligo inhibition and regression.

CHAPTER 3

AIM II: Determine the contribution of B cells to tumor containment and depigmentation.

SUB-AIM I: Determine which immune cells, if any, are involved in tumor containment as a result of HSP70i_{Q435A} vaccination.

From these experiments I am hoping to gain further insight into which immune cells are involved in tumor containment following a vaccination with HSP70i/ HSP70i_{Q435A}. This involves the transfer of cells from vaccinated mice to naïve, tumor-challenged mice. The cells being transferred are CD8⁺ T-Cells, CD11c⁺ Dendritic Cells, and B220⁺ B cells. A second adoptive cell transfer experiment involved the transfer of cells from HSP70i/HSP70i_{Q435A} vaccinated C57BL/6J mice into tumor-challenged immunodeficient SCID-beige mice. SCID-beige mice were used due to their lack of a functional immune system which can help decipher whether an individual cell type is solely responsible for anti-tumor protection during the adoptive cell transfer.

EXPERIMENTAL APPROACH

WT C57BL/6J mice are being used in this experiment; for each experimental group, n=5. There are 2 vaccination groups, 6 recipient groups, and 1 control group. The vaccination groups are prepared and Gene Gun vaccinated for five weeks with either WT HSP70i or HSP70i_{Q435A} (in pUMVC3 DNA vector) as described in **Aim I: Sub-Aim I**.

One week after the final vaccination, these two vaccination groups were sacrificed and their spleens were removed for splenocyte harvest. The spleens were homogenized using a 70µm Nylon Mesh sterile cell strainer and washed with ice-cold HBSS; the spleen homogenates were pooled together according to vaccination group (product is kept on ice during the splenocyte harvest). ACK Lysis Buffer was used to lyse red blood cells from the homogenate and DNase was used to reduce clumping. Cells were washed with ice-cold media then pelleted and resuspended in media. Cells were counted in preparation for sorting with MACS Separation Beads (Magnetic-Activated Cell Sorting). CD11c⁺ Dendritic Cells were sorted out first, followed by CD8⁺ T-Cells, and finally B220⁺ B cells. Using CD11c⁺ DC sorting as an example, the splenocytes were magnetically labeled with magnetic MicroBeads conjugated to anti-CD11c antibodies. The cell suspension is loaded into a column which is attached to a magnetic MACS Separator. The suspension flows through the column and the magnetically labeled cells are retained within the column, while the unlabeled cells flow through. The flow-through is kept on ice for further separation of CD8⁺ T-Cells and B220⁺ B cells. The column is then removed from the magnetic field and the magnetically labeled CD11c⁺ DCs are eluted through and kept on ice. This process is repeated using CD8 MicroBeads and B220 MicroBeads.

At this point, there are 6 cell fractions: DCs, CD8 T-Cells, and B cells from WT HSP70i vaccinated mice as well as DCs, CD8 T-Cells, and B cells from HSP70i_{Q435A} vaccinated mice. The cell fractions are counted, resuspended at 10⁶ cells/100µL, and kept

on ice for immediate retro orbital injection into the recipient mice. The recipient mice are anesthetized using 5% isoflurane and receive 10^6 cells (Cell type is dependent upon their recipient group) via periorbital injection. After cell-transfer injections are complete, the mice were challenged with 10^5 B16 mouse melanoma cells, sub-cutaneously in their right flank. A control group of mice did not receive transferred cells, but did receive the tumor-challenge. Tumor progression was followed as a measure of volume and, upon sacrifice, the tumors were harvested and weighed to measure tumor mass.

RESULTS

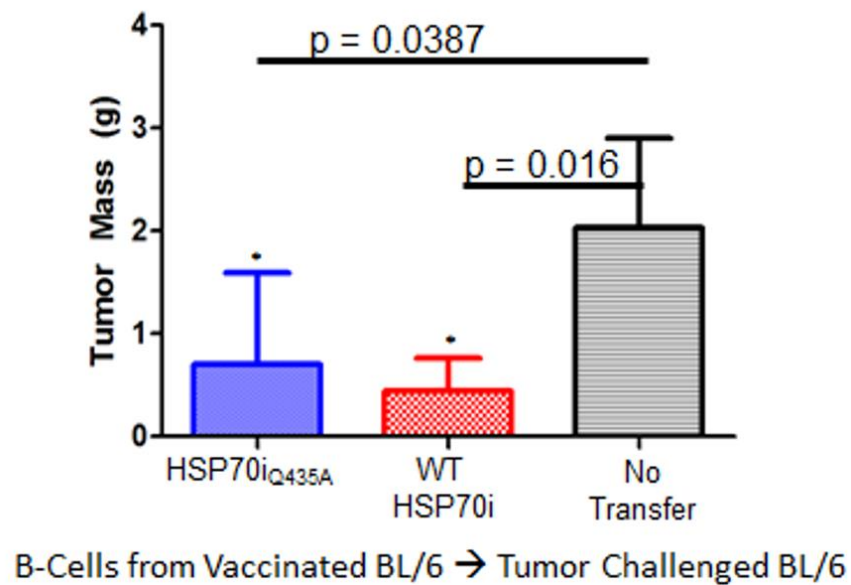


Figure 11: B cell Transfer from vaccinated to tumor-challenged mice confers tumor resistance.

In Figure 11, a student's T-Test revealed that tumor-challenged BL/6 mice that received B cells from the WT HSP70i DNA-vaccinated mice displayed a significant 75% decrease ($p=0.016$) final tumor mass when compared to the average final tumor mass from BL/6 mice receiving B cells from unvaccinated BL/6 mice. Tumor-challenged BL/6 mice that received B cells from Mut HSP70i DNA-vaccinated mice also displayed a

significant 60% decrease ($p=0.039$) in final tumor mass when compared to the average final tumor mass from BL/6 mice receiving B cells from unvaccinated BL/6 mice ($n = 7$ for each group).

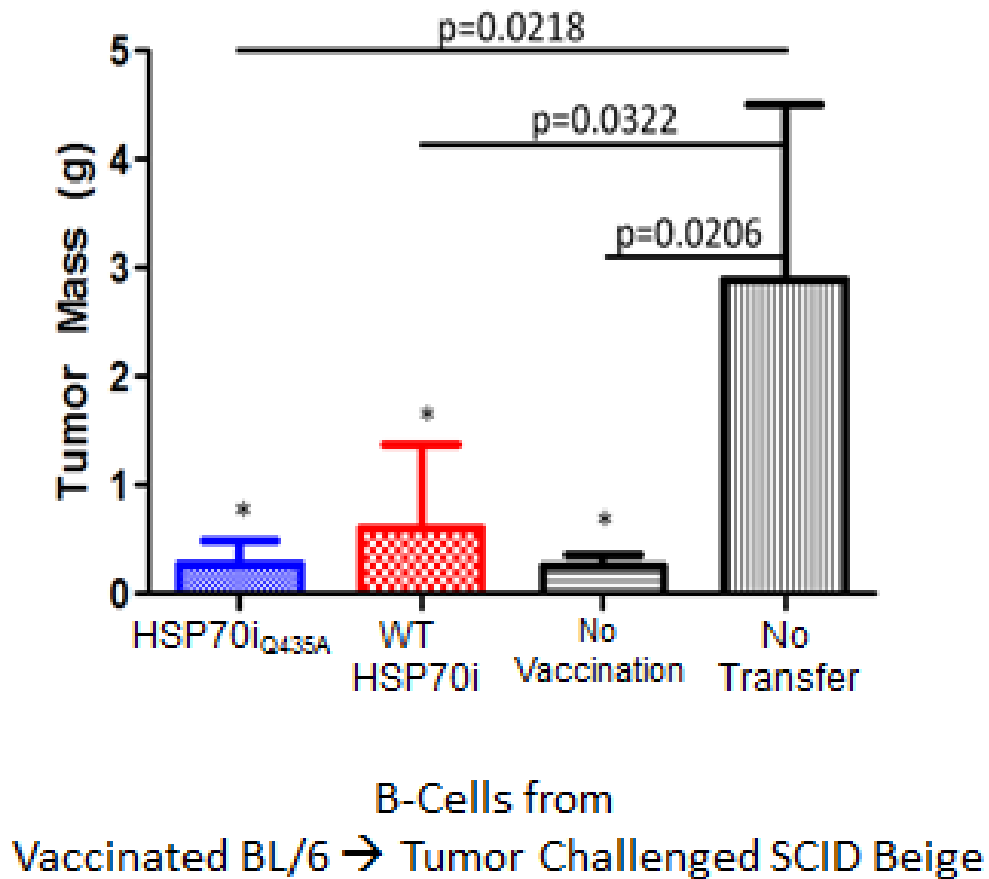


Figure 12: B cell Transfer to tumor challenged SCID/Beige immunodeficient mice confers tumor resistance.

In Figure 12, a student's T-Test revealed a 83% significant decrease ($p=0.032$) in average final tumor mass for the tumor-challenged SCID-beige mice that received B cells

from WT HSP70i DNA-vaccinated BL/6, when compared to the average final tumor mass from SCID-beige mice receiving no cell transfer. Tumor-challenged SCID-beige mice that received B cells from Mut HSP70i DNA-vaccinated BL/6 mice displayed a 90% significant decrease ($p=0.022$) in final tumor mass when compared to the average final tumor mass from SCID-beige mice receiving no cell transfer. Tumor-challenged SCID-beige mice that received B cells from unvaccinated BL/6 mice displayed a 91% significant decrease ($p=0.021$) in final tumor mass when compared to the average final tumor mass from SCID-beige mice receiving no cell transfer ($n = 5$ for each group).

SUB-AIM II: Examine humoral responses to HSP70i after vaccination with the HSP70i_{Q435A} clinical vector.

This Sub-Aim is linked to **Aim I** (**Sub-Aim I**, **Sub-Aim II**, and **Sub-Aim III**) and most of the data generated for this experimental question was analysis from harvested tissue and serum from these previously mentioned mouse/swine vaccination experiments and adoptive transfer experiments.

EXPERIMENTAL APPROACH

At the time of sacrifice, tissue harvesting of mouse skin and tumor were placed in OCT compound and snap-frozen on dry ice, spleens were harvested and homogenized, and a sample of blood was taken. The blood was allowed to coagulate and then spun down 2x to harvest serum.

Swine tissue was collected monthly using a 4mm Acu-Punch biopsy tool; a biopsy was taken from the perilesional region of the vitiligo and a second biopsy was taken from the contralateral flank. 50mls of swine blood was collected bi-weekly; it was allowed to coagulate and then spun down 2x to harvest serum.

For immunohistological staining of tissue sections, 8 μm cryostat sectioned tissues were fixed in cold acetone and stored at $-20\text{ }^{\circ}\text{C}$ until use. Upon use, the tissues were stained using an immunohistological single stain method (using either HRP- or AP-conjugated antibody, and the respective detection solution) or immunofluorescence stain method (using fluorophore-conjugated antibody). The stained tissue sections were imaged on the compound microscope for immunohistological staining or fluorescent microscope for IF staining (at least 7 images taken per slide) and quantified. Mouse tissue was stained with monoclonal, PE-conjugated, hamster IgG antibody specific to the epsilon chain of mouse CD3. Swine tissue was stained with a primary, PE-Cy5-conjugated, mouse IgG1 antibody against the epsilon chain of swine CD3 from manufacturer Abcam; a secondary stain used PE-conjugated, polyclonal rabbit antibody against mouse IgG from manufacturer Dako.

To visualize relative levels of anti-HSP70i in mouse serum after the vaccination experiments, purified HSP70i protein was run through an agarose gel using gel electrophoresis; the protein was then transferred from the gel to a PVDF membrane. Frozen mouse sera from the vaccination experiments outlined in Aim I: Sub-Aim I was

used to probe the membrane, alongside known anti-HSP70i antibodies SPA810 and SPA820. Dako-manufactured polyclonal, AP-conjugated Goat anti-Mouse IgG was used as a secondary to detect bound mouse antibody from the sera probe. AP detection solution was used for development of the membrane and visualization of HSP70i bound mouse antibody.

Humoral responses to autologous and vaccinated HSP70i/HSP70i_{Q435A} will be measured in swine sera by ELISA. To quantify levels of anti-HSP70i antibody in swine serum, an anti-HSP70 ELISA kit from Enzo Life Sciences was used. Swine serum was applied to the HSP70-coated wells, the wells were washed, a secondary, HRP-conjugated Goat anti-Swine IgG antibody was added, and, after washing, the TMB substrate is added to measure concentration of HRP-conjugated secondary bound to anti-HSP70i antibody from swine sera. To quantify levels of HSP70i protein in the swine serum, an HSP70 high sensitivity ELISA kit from Enzo Life Sciences was used. Swine serum was applied to the wells which were coated with anti-HSP70 antibody. After washing, a primary anti-HSP70 antibody was added, the plates are later washed, and an HRP-conjugated secondary antibody specific to the primary antibody is added. The plate is washed and the substrate is added to measure concentration of HRP-conjugated secondary bound to anti-HSP70 primary bound to HSP70 from the swine sera.

RESULTS

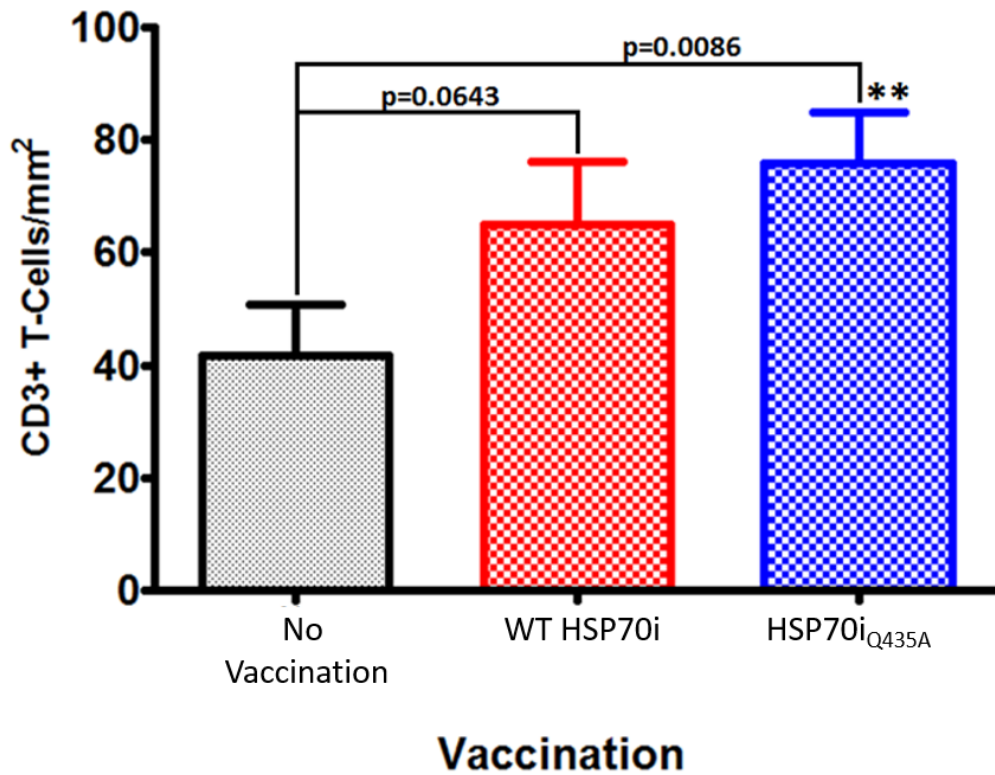


Figure 13: Vaccination with pUMVC3 vector DNA encoding HSP70i WT and HSP70i_{Q435A} promotes T-Cell infiltration.

In Figure 13, the CD3⁺ T-Cell quantification of tumor sections from the vaccination experiment outlined in **Aim I: Sub-Aim I** show a trend toward increased amounts of CD3⁺ T-Cells at the periphery of the tumors of WT HSP70i vaccinated mice (p=0.0643), with a 50% increase in CD3⁺ T-Cells as compared to the quantification of CD3⁺ T-Cells in the tumor tissues from the non-vaccinated mice. A student's T-Test revealed a significant 80% increase (p=0.0086) in CD3⁺ T-Cells at the periphery of the tumors from HSP70i_{Q435A}

vaccinated mice, when compared to the quantification of CD3+ T-Cells at the periphery of tumor tissues from the non-vaccinated mice.

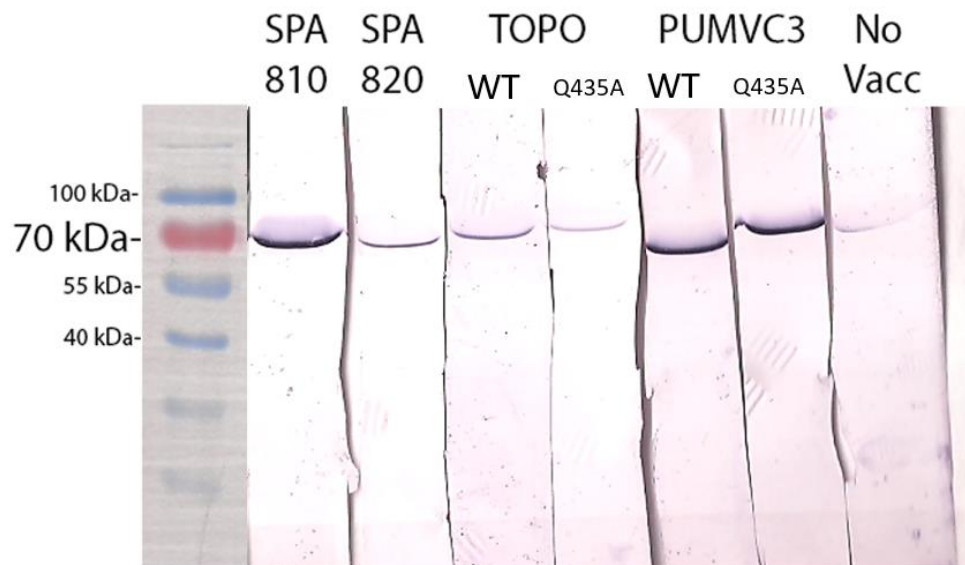


Figure 14: Vaccinated mice produce more robust antibody response toward HSP70i when compared to unvaccinated mice.

Figure 14 shows the results of a Western Blot where serum from vaccination-study mice (**Aim I: Sub-Aim I**) was used as a probing antibody against purified immunoblotted HSP70i protein. Known anti-HSP70 antibodies, SPA-810 and SPA-820, were included as positive controls. When compared to the mice that were not vaccinated, the sera from vaccinated mice seem to contain a very robust antibody response toward HSP70i, around

a 400% increase in band density as measured in ImageJ; this was seen most in the sera taken from mice vaccinated with HSP70i in pUMVC3 vector.

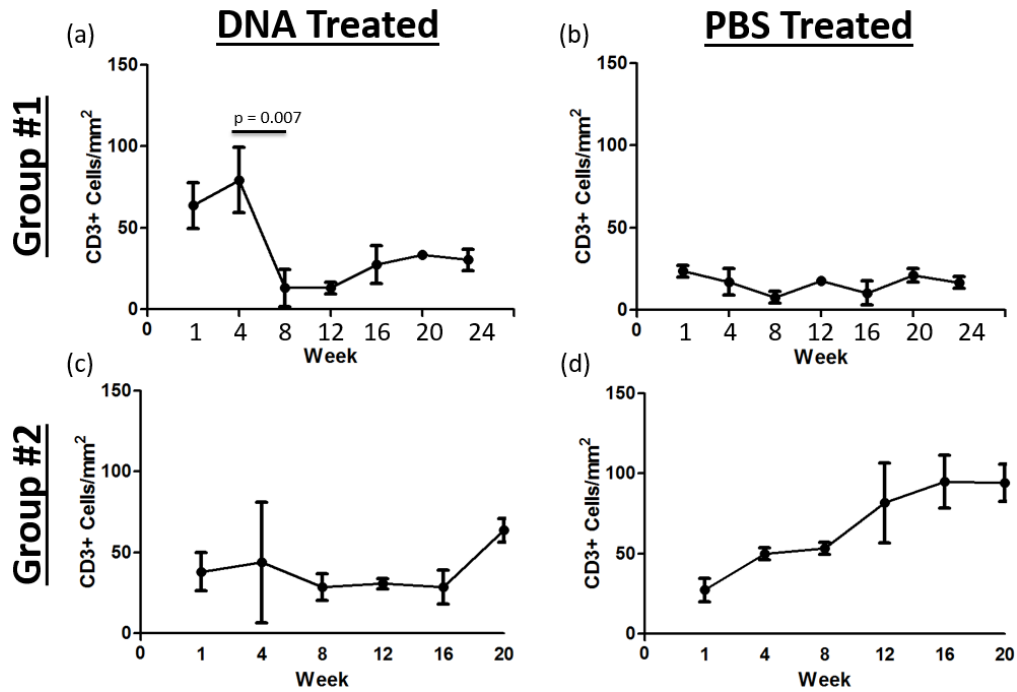


Figure 15: Treatment with DNA-vaccine encoding HSP70i_{Q435A} can inhibit CD3+ T cell infiltration in lesional vitiligo skin.

Swine CD3+ T cells were quantified and measured as CD3+ T cells/mm² of the biopsy area; as we took monthly biopsies, this allowed us follow changes over time in the CD3+ T cell count at the periphery of the lesional areas, as represented in Figure 15. The DNA treated swine from Group #1 exhibits a significant 83% decrease ($p=0.007$) in CD3+ T cell counts between weeks 4 and 8, which coincides with the end of its DNA treatments.

The CD3+ T cell count slowly begins to rise but plateaus at around 30 CD3+ T cells/mm² and does not rise back to original levels observed in the first week [15a]. The PBS treated swine from Group #1 does not display any major increases or decreases in CD3+ T cell counts at the periphery of its melanoma lesional area over the course of 24 weeks [15b]. The DNA treated swine from Group #2 does not display significant changes while the CD3+ T cell counts of the PBS treated swine from group #2 steadily climbs over the course of 20 weeks [15c-d].

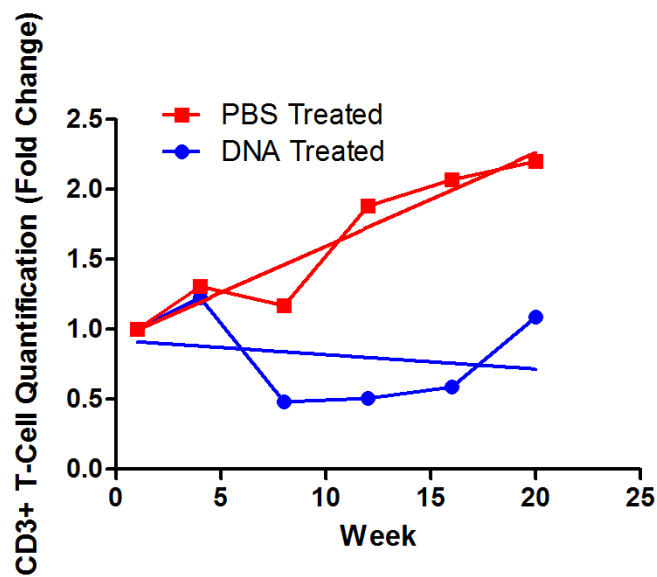


Figure 16: Treatment with DNA-vaccine encoding HSP70i_{Q435A} can inhibit CD3+ T cell infiltration in lesional vitiligo skin over time.

Figure 16 displays the data from Swine Group #2 as the average CD3+ T cell counts of each respective treatment with each week represented as fold change from Week 0.

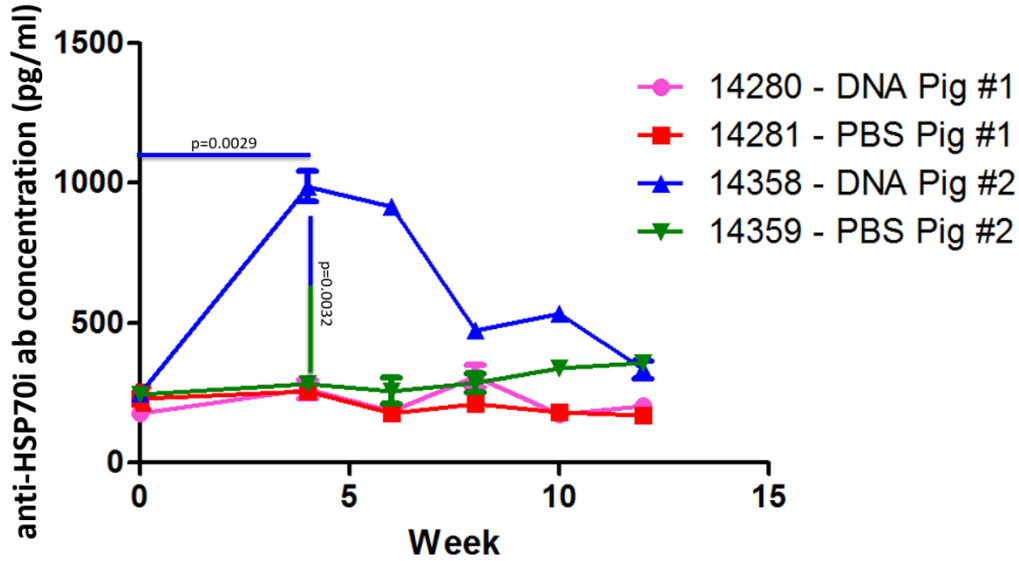


Figure 17: HSP70i DNA-treatment in pigs can produce an antibody response toward HSP70i.

Through an ELISA using sera taken from the swine, it was revealed that the DNA treated swine from Group #2 produced a significant, 300% increase in anti-HSP70i antibody between the first week of treatment and the fourth and final week of treatment; that elevated level of antibody remained into week 6, and began dropping back to basal levels around week 8 as seen in Figure 17. The remaining swine did not display any significant changes in anti-HSP70i antibody concentration.

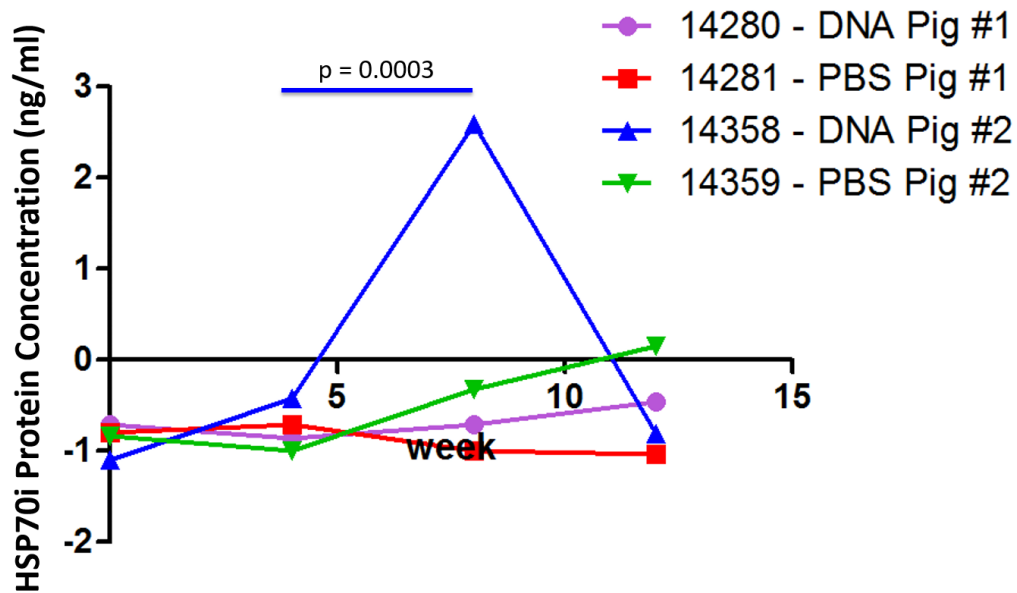


Figure 18: HSP70i DNA-treatment in swine can raise serum levels of HSP70i protein beyond basal levels.

Figure 18 shows the results of an ELISA run with sera taken from the swine to detect serum level HSP70i protein in the swine vaccinated with HSP70i_{Q435A} DNA vaccine. It was revealed that the DNA treated swine from Group #2 produced a significant increase ($p=0.0003$) in serum HSP70i protein between week 4 and week 8, which then dropped back down to basal levels by week 12. The remaining swine did not display any significant changes in serum HSP70i protein concentration.

CHAPTER 4

DISCUSSION

Because HSP70i_{Q435A} inhibits the T cell-mediated auto-immune response against melanocytes in vitiligo mouse models, there existed a valid concern that treatment with this modified heat shock protein may also inhibit natural responses against melanocyte-derived melanoma tumor cells. As such, the first part of this project was to study the effects of HSP70i_{Q435A} DNA-treatment in a mouse melanoma model. The results from these first two mouse studies indicated that not only was HSP70i_{Q435A} not inhibitory of anti-melanoma responses in mice, but DNA-treatment with both WT HSP70i and HSP70i_{Q435A} confer some degree of tumor resistance [Figure 1-3]. Regarding HSP70i_{Q435A} as a therapeutic agent for vitiligo treatment, these results are promising; vitiligo therapy would be a step in the wrong direction if it decreased the patient's immune response to melanoma. This is especially relevant considering vitiligo is oftentimes a side-effect of melanoma therapy as treatments targeting melanoma cells also collaterally target melanocytes; as a result, vitiligo during melanoma therapy is sometimes considered a good sign that the treatment is working (17). Staining results from these initial experiments also show increased CD3 T cell counts around the periphery of the melanoma tumor from mice that received either the WT HSP70i DNA-vaccination or the

HSP70i_{Q435A} when compared to the mice that received no treatment [Figure 13]. This is compared to previous studies which observed decreased T cell infiltration in healthy and vitiligo-affected skin after mice were vaccinated with HSP70i_{Q435A} (Reference 4, Figure 3). These results suggest that DNA-treatment with HSP70i_{Q435A} can promote T cell presence at the melanoma, while decreasing T cell presence at lesional vitiligo skin. Taken together, this information seems to imply separate mechanisms by which HSP70i_{Q435A} DNA-treatment might be inhibiting the auto-immune response against melanocytes and promoting the anti-tumor response to melanoma.

Knowing that melanoma and other tumor cells overexpress and externally present stress induced heat shock proteins such as HSP70i (7), we wanted to test the antibody response to purified HSP70i protein after vaccination with either the WT HSP70i or HSP70i_{Q435A}. After running a Western with purified HSP70i and probing with sera harvested from the mice in the previous experiments outline in Aim I: Sub-Aim I, the results suggested that a DNA-treatment with WT HSP70i and HSP70i_{Q435A} can promote production of antibody against HSP70i and as such may be promoting anti-melanoma responses via antibody-dependent cell-mediated cytotoxicity (ADCC). Previous studies have targeted membrane-bound HSP70 as a means of inducing ADCC killing of tumor cells, where tumor growth was reduced in mice treated with antibody specific to an HSP70 epitope frequently detected on the surface of tumor cells (18). Results from the adoptive transfer experiment outlined in Aim II: Sub-Aim I seem to coincide with those studies and

suggest that antibody-mediation may indeed be the cause of this tumor inhibition. Tumor challenged BL/6 mice that received B cells from HSP70i DNA-vaccinated BL/6 mice displayed markedly smaller tumors, suggesting the B cell transfer was responsible for the tumor inhibition [Figure 11]. To further test this, B cells taken from vaccinated BL/6 mice were adoptively transferred into tumor-challenged, immuno-deficient SCID-beige mice. Strangely, however, results showed decreased tumor growth in all mice that received B cell injections, even the mice that received no HSP70i DNA-treatment [Figure 12]; however, there was no significance between the vaccinated and unvaccinated tumors. Considering that SCID-beige strain is immuno-deficient of B and T cells, while still containing defective NK cells, it might be possible that antibody from these adoptively transferred B cells may still be enough to activate the NK cells in SCID-beige mice (although this does not explain why the adoptive transfers from non-vaccinated mice are doing so). Adoptive transfers of sensitized and primed B cells have previously been shown to promote humoral anti-tumor responses in mice, where the adoptive transfer resulted in production of tumor-specific antibody and tumor cell lysis in the presence of complement (19). As SCID-beige mice contain normal complement levels, it is likely that complement-dependent cytotoxic killing of tumor cells played a role in both of the adoptive transfers.

A separate vaccination and tumor challenge experiment was also done on PMEL-1 mice. These mice were chosen as they have transgenic T cell receptors specific to the

mouse homologue (pmel-17) of human gp100, a melanocytic protein involved in pigment synthesis. They naturally depigment which makes this strain a good choice for a vitiligo mouse model; however it can take up to several months for any natural depigmentation to occur. For this reason, the mice used in Aim I: sub-aim II were also vaccinated with the DNA encoding gp100 to facilitate and speed up the depigmentation, alongside either the WT HSP70i DNA and HSP70i_{Q435A} DNA. Results from the tumor growth and final tumor mass once again suggest anti-tumor responses mediated by treatment with an HSP70i DNA-vaccine are not affected by the _{Q435A} mutation in the stress protein [Figure 6, Figure 5]. As for the depigmentation results, mice treated with DNA encoding HSP70i_{Q435A} display slightly decreased average depigmentation at the end of the experiment when compared to mice treated with DNA encoding WT HSP70i [Figure 7, Figure 8]. These data don't quite coincide with data from previous depigmentation studies, where the depigmentation was significantly decreased in mice treated with DNA encoding HSP70i_{Q435A}; it's likely that the addition of DNA encoding gp100 during the gene-gun vaccinations may have caused this inconsistency and largely overruled the dampening effects of HSP70i_{Q435A}, facilitating depigmentation a lot more than we originally bargained for. Future studies will likely omit or reduce the gp100 treatment, assuming that time is not a constraint as it was during this study. Additionally, this study where treatment with gp100 is done alongside HSP70i_{Q435A} may prove useful in future trials related to gp100 treatment; as clinical trials have already demonstrated gp100 as a successful melanoma antigen to target for

melanoma therapy (20, 21) there will likely be a demand for secondary or additional primary treatments to deter the vitiligo that develops as a result of the patient's immune system targeting melanocytic gp100.

To set the stage for further developments of HSP70i_{Q435A} as a treatment for autoimmune side effects of melanoma immunotherapy, we chose to test the efficacy of these treatments on a swine model of vitiligo. This substrain of Sinclair miniature swine is unique in that the swine spontaneously develop melanoma at a young age and, as the melanoma regresses, they develop vitiligo as a side-effect (usually seen as a ring of depigmented skin around the regressing melanoma). It should also be noted that 2 out of the 4 swine that were sent to us did not have visible lesions of vitiligo which complicated things in terms of analyzing the results. We used the DNA treatment on the 2 swine that had visible vitiligo, and the PBS control treatment on the two swine that did not have visible vitiligo (PBS injections were performed at a site of melanoma on these 2 swine). The data seems to indicate a positive response from the DNA treatment in the first swine [Figures 15a, 17a, 18ab] while there was not as much of a response in the second DNA treated swine [Figures 15c, 17c, 18cd]. However, we must also consider that any area of regressing vitiligo would see increasing levels of infiltrating T cells, and the maintenance of a steady level in DNA Swine #2 may be indicative of a positive response to the treatment. These swine are incredibly diverse when compared to a strain of lab mouse, and variation in response to any experimental treatment is not unexpected. However, it

is very promising to see the treatment working the way it is meant to be working in the first DNA treated pig, and watching the T cell counts around the lesion decrease as the lesion itself decreases in size and begins to repigment. Furthermore, one of the milestones we had set for the trial included a 50% reduction in T cell infiltration which was observed in the Group #1 swine treated with DNA, with a 75% decrease in CD3+ T cells between the first week and the eight week; even comparing the first week with the final week, there is still a 58% decrease [Figure 15a]. The data from the two ELISAs [Figures 17, 18] seems to suggest that the second swine did respond but in a different manner than the first swine; where the first swine managed to divert the cytotoxic response away from the treated area, the second swine began producing antibody against HSP70i. Unfortunately, it's still too early to judge what effect this might have on the swine's anti-melanoma responses but it will definitely be interesting to follow.

Conclusive Remarks

The results from these studies support the ongoing development of a novel therapeutic that not only has the ability to restrict with the autoimmune responses that cause vitiligo, but also maintain anti-tumor responses at the same time. Treatment with HSP70i_{Q435A} helps inhibit the cytotoxic auto-immune response against healthy melanocytes, does not interfere with anti-tumor responses to melanoma cells, and also seems to support anti-melanoma responses by stimulating production of anti-HSP70i antibody which are able to direct antibody dependent cell-mediated cytotoxic responses

against the HSP70i-saturated melanoma cells; these mechanisms are summarized in Figure 19.

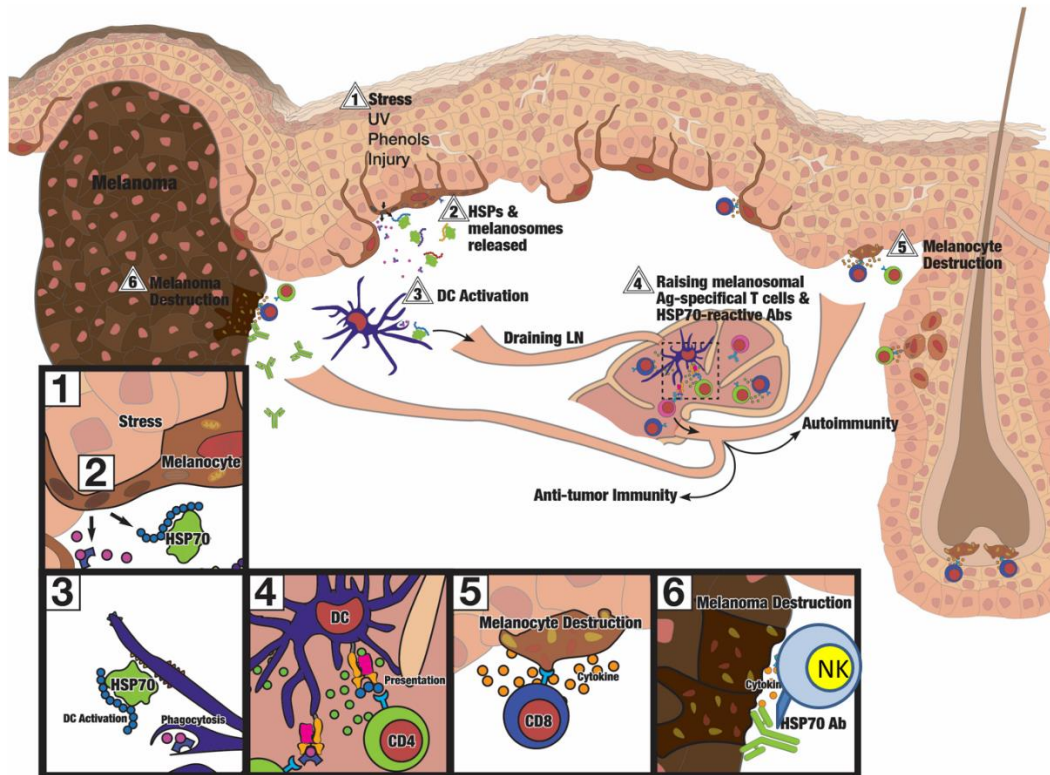


Figure 19: Mutant HSP70i_{Q435A} promotes anti-tumor immunity while preventing autoimmunity.

(1) In vitiligo, stress to healthy melanocytes induces synthesis of HSP70i which is (2) secreted from live cells through an active-release mechanism. These secreted HSP70i proteins can potentially be chaperoning misfolded melanocytic proteins that are in the process of refolding within the cell and, once in the extracellular environment, (3) bind to and activate local dendritic cells to an inflammatory phenotype. These dendritic cells

move through the draining lymph nodes where they (4) present melanocyte antigen to T cells and stimulate a (5) melanosomal-ag specific CD8+ T cell cytotoxic response that results in melanocyte destruction and depigmentation characteristic of vitiligo. When HSP70i_{Q435A} is introduced to the early steps of the system, a single amino acid change (Glutamine to Alanine) in the DC-binding region of HSP70i is sufficient to deter (3) DC activation to an inflammatory phenotype, instead inducing a non-inflammatory phenotype which do not process and present melanocyte antigen. B cells in the lymph are presented with DC-bound HSP70i and, upon recognition, proliferate and produce HSP70i-specific antibody. (6) These antibodies recognize and bind surface HSP70i on melanoma cells; CD16+ natural killer cells then bind the Fc region of the antibody, stimulating the NK cell to release perforin, forming lytic pores in the melanoma, and granzyme to induce apoptosis in the targeted melanoma cell.

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VITA

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In August of 2013 he joined the Microbiology and Immunology program at Loyola University Chicago. In January of 2013 he joined the laboratory of Dr. Caroline Le Poole where he studied the potential therapeutic role of heat shock proteins in the treatment of vitiligo and melanoma. His studies focused primarily on a stress-induced 70kDa Heat Shock Protein (HSP70i) that is known to play a role in triggering the auto-immune response against healthy melanocytes during progression of the vitiligo disease state, and how it might have therapeutic significance in treating cancers such as melanoma. His work on HSP70i was presented at the 22nd International Pigment Cell Conference 2014 in Singapore.

After completion of his M.S. at Loyola he plans to find a position at a research lab within the Biomedical Research industry.