MUTANT HSP70I TO PREVENT AUTOIMMUNE DISEASE

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A vaccine and method of treatment suitable for treating autoimmune diseases, such as Vitiligo, by using variant peptides representing a sequence of amino acids found in heat shock protein 70. The vaccine includes a peptide derived from inducible heat shock protein 70 and a plasmid containing a full inducible heat shock protein 70 DNA sequence encoding the peptide.
FIG. 4A

CD3 cells/mm²

Strain: C57BL/6 hsp70-1 KO
Vaccc: TRP-1 + HSP70i TRP-1 + HSP70i

* *

FIG. 4B

TRP-1 Stained Follicles/mm²

Strain: C57BL/6 hsp70-1 KO
Vaccc: TRP-1 + HSP70i TRP-1 + HSP70i

* *
FIG. 7
**FIG. 8**

Bar charts comparing depigmentation relative to control for different genotypes and treatments. The charts show data for pmel-1 and C57BL/6 mouse strains, with comparisons between WT, MT, and Vector groups. The data is presented in terms of percentage depigmentation relative to control (Dorsal and Ventral). The charts include statistical significance indicators (*** and **) for comparisons between groups.
FIG. 11
CD8 KO Mice

Empty Vector + TRP-1

HSP70 (0.435A) + TRP-1

Wild-type HSP70 + TRP-1
Day 13 tumor growths

Tumor volumes (cm$^3$)

FIG. 12
CD8 KO Empty Vector + TRP-1
CD8 KO Mutant 8 + TRP-1
C57BL/6 Empty Vector + TRP-1
C57BL/6 Mutant 12 + TRP-1

Days post injection

Tumor Volume (cm^3)

FIG. 13
MUTANT HSP70I TO PREVENT AUTOIMMUNE DISEASE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/292,050, filed Aug. 30, 2011, the contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] The present invention generally relates to treatments for autoimmune diseases. More specifically, this invention relates to the use of variant peptides representing a sequence of amino acids found in heat shock protein 70 (HSP70) to treat autoimmune diseases such as Vitiligo.

[0003] Vitiligo is an autoimmune disorder of the skin presenting with depigmenting lesions, affecting approximately 0.5% of the world’s population. Vitiligo lesions are milky-white patches that can increase in shape and size over time and the distribution of these lesions defines disease classification. In particular among dark skinned individuals, emotional effects can range from mild embarrassment to severe social anxiety reinforcing the impact of Vitiligo on a patient’s quality of life. Meanwhile, treatment opportunities are very limited and largely unsuccessful.

[0004] Support for Vitiligo as an autoimmune disease is provided by infiltrates of T cells and macrophages described for skin of actively depigmenting patients. Circulating autoantibodies towards melanocyte antigens have also been detected. Mutations in genes associated with autoimmunity have been identified by family studies, such as a human leukocyte antigen (HLA) system which defines antigen presentation, and Vitiligo melanocytes often display anomalies in gene expression of proteins essential in antigen processing and protein folding. In addition, Vitiligo patients often develop other autoimmune disorders such as thyroid disease, pernicious anemia, Addison’s disease, and lupus. These and several other autoimmune disorders have been linked with HLA molecules, particularly HLA class II (HLA-DR). Patients with HLA molecules susceptible to binding autoantigens have higher frequencies of autoreactive T cells in their circulation. As often observed in autoimmune-affected tissue, peri-lesional skin from Vitiligo patients displays elevated expression of HLA-DR.

[0005] Immunohistochemical staining of Vitiligo skin with a panel of antibodies reactive with different melanocyte-specific features has revealed in previous studies that depigmentation is due to a loss of melanocytes rather than a disruption of melanin production. The role of such infiltrates in mediating an immune response specifically to melanocytes is supported by increased frequencies of T cells reactive with melanocyte antigens in both the skin and circulation of Vitiligo patients. Increased infiltration of T cells in Vitiligo skin involves primarily CD8+, that is, cytotoxic T cells pointing to a Th1-mediated immune response.

[0006] Patients frequently indicate that their Vitiligo lesions were augmented or initiated under the influence of stress, including triggering factors such as UV overexposure, mechanical trauma or contact with bleaching phenols. Psychological stress may also contribute to Vitiligo development. Such precipitating factors then translate into an autoimmune response specifically targeting melanocytes. To further understand this, stress can directly affect the immune system, as evidenced by changes in CD4+/CD8+ T cell ratios following stress mediated activation of R-adrenergic receptors of the sympathetic nervous system. Upon activation of the sympathetic nervous system, heat shock proteins (HSP) released into the extracellular milieu can drive the immune response. Thus HSPs can mediate the translation between causative stress factors and the autoimmune response to follow.

[0007] HSP70 is a family of proteins including constitutive HSP70 and inducible HSP70 (HSP70i). HSP70i is among cytoplasmic chaperones upregulated in response to temperature changes and other forms of stress. HSP70i is involved in maintaining molecular and cellular integrity, preventing protein misfolding and subsequent apoptosis. Extracellular HSP70i in turn serves as an alarm signal to the immune system by supporting uptake, processing and presentation of chaperoned antigens in the context of MHC class I and II, and invoking both the innate and adaptive responses. Dendritic cell (DC) activation allows for subsequent recruitment of CD8+ T cells from draining lymph nodes reactive with chaperoned proteins and peptides.

[0008] The unique role of HSP70i in precipitating autoimmunity may be better understood considering that HSP70i is actively secreted by living cells. Melanocytes established from non-lesional Vitiligo skin treated with skin bleaching 4-tertiary butyl phenol (4-TBP) has been shown in previous studies to increase secretion of HSP70. The same stress also confers sensitivity to DC-mediated cytotoxicity through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). HSP70i is released into the circulation via necrosis, or secreted by active transport through exosomal release. Similar to the proposed mechanism for inducing autoimmunity in Vitiligo, in vivo studies have shown that HSP70i secreted from tumors confer anti-tumor responses via the peptides they chaperone.

[0009] The role HSP70 has on the immune response can in part be explained by the activation of DCs. This is supported by evidence that HSPs chaperoning antigenic peptides can induce DC-mediated activation of cytotoxic T lymphocytes (CTLs). In addition, HSPs enhanced the presentation of tyrosinase peptide to specific CTLs. HSP70 also activates DCs directly, as indicated by upregulation of the maturation markers CD40, CD83, and CD86. A 20-mer peptide sequence within microbial HSP70 has been assigned an immune regulatory function. HSP70 is believed to bind multiple receptor molecules on the DC membrane including TLR4, TLR2, CD14, CD91, and CD40. Antibodies that bind these receptors are generally capable of activating DCs as well. Thus, inhibiting DC activation by blocking receptors from binding HSP70 is not expected to meet with success as a measure to prevent autoimmune responses.

[0010] In view of the above, it can be appreciated that improved methods of treating patients with autoimmune diseases, such as Vitiligo, are needed.

BRIEF DESCRIPTION OF THE INVENTION

[0011] The present invention provides a vaccine and method of treatment suitable for treating autoimmune diseases, such as Vitiligo, by using variant peptides representing a sequence of amino acids found in heat shock protein 70 (HSP70).

[0012] According to a first aspect of the invention, a vaccine for treating an autoimmune disease includes a peptide derived from inducible heat shock protein 70 and a plasmid
containing a full inducible heat shock protein 70 DNA sequence encoding the peptide.

[0013] According to a second aspect of the invention, a method of treating an autoimmune disease includes deriving a peptide from an inducible heat shock protein 70, forming a vaccine including the peptide derived from inducible heat shock protein 70 and a plasmid containing a full inducible heat shock protein 70 DNA sequence encoding the peptide, and then vaccinating a human patient with the vaccine.

[0014] A technical effect of the invention is the ability to treat autoimmune diseases, such as vitiligo by reducing activation of dendritic cells. In particular, it is believed that, treating a patient with a vaccine comprising a variant peptide representing a sequence of amino acids found in heat shock protein 70 (HSP70) will reduce activation of dendritic cells and thereby reduce the symptoms of autoimmune diseases.

[0015] Other aspects and advantages of this invention will be better appreciated from the following detailed description.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0016] FIG. 1 is a scanned image showing C57BL/6 wild-type (wild-type) mice and inducible heat shock protein 70 knockout (HSP70-1 KO) mice that were gene gun vaccinated with either antigenic Tyr1Lee (TRP-1) or TRP-1 plus inducible heat shock protein 70 (HSP70) and imaged 4 weeks after the final vaccination.

[0017] FIG. 2 is a graph representing image analysis of depigmentation in the wild-type mice and the HSP70-1 KO mice of FIG. 1.

[0018] FIG. 3A is a pair of graphs represent fluorescence-activated cell sorting (FACS) used to determine peptide-specific cytotoxic T lymphocyte (CTL) activity for in vivo cytotoxicity assays.

[0019] FIG. 3B is a graph representing cytotoxicity in the mice of FIG. 3A.

[0020] FIGS. 4A and 4B are scanned images showing skin near the dermo-epidermal junction from wild-type mice and HSP70-1 mice one week after a final booster gene gun vaccination and their respective graphs representing quantification of T cell infiltration. CD3 T cells are represented by solid arrows, hair follicles are represented by asterisks (*) and gold particles are represented by open arrows.

[0021] FIG. 5A is a scanned image of wild-type mice and constitutive HSP70 knockout mice (HSP70-2) that were gene-gun vaccinated with either TRP-2 or control DNA (plasmid containing no gene insert).

[0022] FIGS. 5B and 5C are graphs representing the depigmentation and cytotoxicity, respectively, observed in the mice of FIG. 5A.

[0023] FIG. 6A is a Western Blot representing HSP70i protein expression and antibody recognition.

[0024] FIG. 6B is a graph representing image analysis of depigmentation in mice vaccinated with mutant versions of HSP70i and TRP-2 as compared to wild-type HSP70.

[0025] FIG. 7 is a scanned image of wild-type and Vitiligo-prone, 78B6.Cg-H2b1a/Cy Tg(TcraTcrgb8Rest1+/J gp100 T cell receptor transgenic (pmel-1) mice that were vaccinated with either wild-type (WT), HSP70i (Q435A) (MT), or control DNA (vector) three months after the final gene gun vaccination.

[0026] FIG. 8 is a pair of graphs representing quantification of depigmentation of the mice of FIG. 7 relative to control DNA.

[0027] FIGS. 9A and 9B are graphs representing quantification of splenocytes obtained from pmel-1 mice nine months after gene gun vaccination with wild-type (WT) or mutant HSP70i (Q435A) (MT), and control DNA (vector) and were stained to discriminate leukocyte subpopulations, and staining was quantified by flow cytometry.

[0028] FIGS. 10 and 11 are scanned images representing wild-type mice and CD8 knockout mice that were vaccinated with either control DNA and TRP-1, HSP70i (Q435A) and TRP-1, or wild-type HSP70i and TRP-1.

[0029] FIGS. 12 and 13 are graphs representing observed tumor growth in the mice of FIGS. 10 and 11.

[0030] FIGS. 14 and 15 are graphs representing quantification of monocytes harvested and magnetically sorted from peripheral blood of the mice of FIGS. 10 and 11 and stained for CD11C, CD80, CD86, CD83, HLA-DR, HLA-DR, all directly with conjugated fluorochromes.

[0031] FIG. 16 is a scanned image of a wild-type mouse and a pmel-1 mouse and their respective skin samples prior to testing.

**DETAILED DESCRIPTION OF THE INVENTION**

[0032] The present invention will be described in terms of methods for treatment of autoimmune diseases. In one aspect of the invention, a mutant inducible heat shock protein 70 is used to treat depigmentation resulting from the autoimmune disease Vitiligo.

[0033] Vaccines including melanocyte-specific antigens in combination with HSP70 have been shown in previous studies to elicit immune responses towards tumorigenic and healthy melanocytes. Whereas intracellular heat shock proteins serve a cytoprotective function, upon secretion, an inducible isoform of HSP70 (HSP70i) functions as a chaperone, eliciting innate and adaptive immune responses to surrounding tissue. Under stressful conditions, secreted, HSP70i is believed to serve as an adjuvant in Vitiligo, binding melanocytic antigens to be processed by antigen presenting cells (APC) and eliciting autoimmune responses to melanocytes in active disease. HSP70i may also activate dendritic cell (DC) directly through specific receptor binding, leading to production of various cytokines such as IL-12, and IFN-γ and the subsequent activation and recruitment of effector cells from draining lymph nodes. Thus, the identification of a peptide within HSP70i crucial to eliciting autoimmunity would open doors to preventing the causative chain of events leading to depigmentation in Vitiligo.

[0034] As previously discussed, heat shock protein 70 (HSP70) as well as various other heat shock proteins, have been implicated in DC activation. It is believed that inhibiting DC activation by blocking receptors from binding HSP70 is not expected to meet with success as a measure to prevent autoimmune responses. Therefore, moieties within HSP70 capable of binding and activating DCs were investigated in order to modulate the development of autoimmune Vitiligo. Specifically, HSP70i was investigated to determine whether its role in Vitiligo is unique or redundant in order to justify targeting a single stress protein as a treatment measure for autoimmune disease. HSP70i was chosen for its advantages over other heat shock proteins. For example, unlike other heat shock proteins, HSP70i is secreted by living cells, thus its actions occur naturally in vivo. In addition, as compared to other heat shock proteins, HSP70i is more effective at eliciting immune responses.
The following trials were conducted using C57BL/6 wild-type (wild-type) mice, HSP70-1 knockout (HSP70-1 KO) mice, constitutive HSP70 knockout (HSP70-2 KO) mice, and Vitiligo-prone, 78B6.Cg-Thy1a/Cy 7tg(TcraTcrb)8Rest/J gp100 T cell receptor transgenic (pmel-1) mice. All mice were six to eight weeks of age when included in experiments, excluding the HSP70-2 KO mice which were between ten to fourteen months of age, and all mice were gender matched to wild-type mice. The mice were maintained in facilities approved by institutional IACUC regulations.

A first trial was performed to assess whether there is a sole requirement for HSP70 in mediating depigmentation. Wild-type mice and HSP70-1 KO mice were gene gun vaccinated with antigenic Tyr lasting (TRP-1) encoding plasmid. The mice were gene gun vaccinated five times every six days with DNA encoding eight or four optimized TRP-1 or three TRP-1 plus HSP70-1 KO mice. Image analysis confirmed significant depigmentation in the wild-type mice after vaccination with optimized TRP-1, indicating that expression of HSP70 is required for efficient induction of autoimmunity, as represented in the chart of FIG. 2. These data demonstrated that the human HSP70 encoding plasmid in the vaccine is insufficient to restore the depigmentation process in the HSP70-1 KO mice. Therefore, it is believed that depigmentation is the result of HSP70-enhanced DC function, accompanied by activation of T cells specifically reactive with melanocytes.

To determine if HSP70 mediates the immune activation of cytotoxic T cells (CTL), wild-type mice and HSP70-1 KO mice vaccinated with a combination of optimized TRP-1 and HSP70-1 encoding plasmids were assessed for in vivo cytotoxicity towards the encoded antigen as well as reactivity towards tyrosinase-related protein 2 (TRP-2). For in vivo cytotoxicity assays, the mice were challenged with splenocytes pulsed with either TRP-1, TRP-2, or irrelevant control peptides plus differing concentrations of carboxyfluorescein succinimidyl ester (CFSE). The mice received two booster gene gun vaccinations three days apart. Spleens were harvested 18 hours after challenge, and fluorescein-activated cell sorting (FACS) was used to determine peptide-specific CTL activity. Data from individual wild-type mice and HSP70-1 KO mice are represented in FIGS. 3A and 3B. The wild-type mice displayed significantly increased killing of splenocytes pulsed with a peptide derived from TRP-1 as compared to the HSP70-1 KO mice following gene gun vaccination. That is, the wild-type mice depigmented significantly more as compared to the HSP70-1 KO mice after each vaccination. The data also revealed cytotoxicity towards the TRP-2 peptide-pulsed splenocytes in all groups, indicating that epitope spreading had occurred. A correlation was observed between increased depigmentation and cytotoxicity towards splenocytes, both of which are augmented by HSP70. In all, data confirm a solid requirement for HSP70 in inducing CTL mediated autoimmune Vitiligo and showed that the HSP70-1 mice are resistant to cytotoxic depigmentation after TRP-1 vaccinations.

To further support the belief that HSP70-induced skin immune reactivity is responsible for the Vitiligo phenotype, local differences in immune reactivity in response to HSP70 were investigated. Skin from wild-type mice and HSP70-1 KO mice were probed one week following booster gene gun vaccinations using antibodies against pan T lymphocyte marker CD3 and the density and location of the stained cells was evaluated. FIG. 4A shows a scanned image of skin samples near the dermo-epidermal junction from the wild-type mice containing an increased number of CD3+ T cells (indicated by solid arrows) as compared to the HSP70-1 KO mice one week after a final booster gene gun vaccination. CD3+ T cells are more abundant than hair follicles (indicated by *) in the wild-type mice. Gold particles can also be observed (indicated by open arrow). FIG. 4A further includes a graph representing quantification of T cell infiltration within the skin samples. The vaccinated skin of the wild-type mice contained significantly more T cells than the HSP70-1 KO mice. Consistent with depigmentation data, immunodetection of melanocyte antigen TRP-1 indicated less melanocyte-containing hair follicles were maintained within the wild-type mouse skin as compared to skin from the HSP70-1 KO mice. FIG. 4B is a scanned image showing skin samples with more melanocyte-containing hair follicles in the HSP70-1 KO mice as compared to the wild-type mice after vaccination. TRP-1 expressing melanocytes (indicated by solid arrows) are shown within hair follicles (indicated by *) in the HSP70-1 KO mouse. FIG. 4B further includes a graph representing quantification of melanocyte containing follicles. Depigmentation is shown to correlate with peptide-specific CTL activity, supporting the causative involvement of CD8+ T cells. Taken together, these immunohistological data firmly establish HSP70 induced Vitiligo developing in the skin of depigmenting mice indicating that HSP70 is necessary for loss of melanocytes by T cells.

In an additional trial, HSP70 was confirmed to be a sole isoform responsible for accelerating autoimmune depigmentation by gene gun vaccinating HSP70-2 KO mice with TRP-2 encoding plasmid in concentrations sufficient to induce depigmentation in wild-type mice. The wild-type and the HSP70-2 KO mice were gene-gun vaccinated five times every six days with six or eight optimized TRP-2 or control DNA (plasmid containing no gene insert). FIG. 5A is an image representing depigmentation in the wild-type mice and the HSP70-2 KO mice. Unlike the above trials engaging the HSP70-1 KO mice, no difference in depigmentation was observed in both the wild-type and the HSP70-2 KO mice. Conversely, similar amounts of depigmentation were observed in both the wild-type and the HSP70-2 KO mice vaccinated with TRP-2 as compared to control DNA vaccinations. Visual observations were supported by statistical analysis of quantified data, as represented in the graph of FIG. 5B.

In addition, a graph of FIG. 5C represents that there were no differences in cytotoxicity towards peptide-pulsed splenocytes from the wild-type mice and the HSP70-2 KO mice following gene gun vaccination with the TRP-2 encoding plasmid. This is unlike the HSP70-1 KO mice which displayed less killing towards the vaccinated antigen (TRP-1). Together, these data show that constitutive HSP70 is irrelevant for inducing an autoimmune response and further confirm the unique requirement for HSP70 expression in precipitating autoimmune Vitiligo.

Due to the results of the above trials, mammalian HSP70 was investigated to determine if it contains a peptide moiety responsible for binding and activation of DC that may be targeted to modulate autoimmune depigmentation. A database search identified the peptide QPGV1. IQVYEG3ER in human HSP70 as maximally homologous with a DC binding region of microbial HSP70. To assess whether this region is indeed crucial for the autoimmune activation, mutations were
inserted via site directed mutagenesis, results of which are represented in a Western Blot of FIG. 6A.

[0042] For site directed mutagenesis human and microbial inducible HSP70i were aligned in a BLAST search to identify a 13 amino acid stretch that was maximally homologous to the microbial DC peptide as the putative DC binding domain within human HSP70i. Vectors were created with single or double mutations in the putative DC binding region of human HSP70i (HSP70i(435-447)) using appropriate primers to induce mutations in a sequence QPGVLIQVYEGER. As a template for the site directed mutagenesis, an expression vector encoding HSP70i was used as described. In vitro mutagenesis of HSP70i residues was performed on double-stranded template using polymerase chain reaction as previously described. Mutant sequences were verified by DNA sequencing. To verify protein expression, COS7 (CV-1 (simian) in origin, and carrying SV40 genetic material) cells were transfected with individual plasmids encoding human HSP70i, or the mutant HSP70i plasmids using lipofectamine and verified protein expression by Western blotting. Blots were probed with anti-HSP70 Ab (rabbit polyclonal Ab SPA-811 or mouse mAb SPA-810) and alkaline phosphatase-conjugated secondary Abs (goat anti-rabbit, or goat anti-mouse).

[0043] A mutant plasmid HSP70i(Q435A) is recognized equally efficiently as wild-type HSP70i whereas monoclonal antibody SPA-810 shows 20-30% reduced reactivity towards the mutant HSP70i plasmids HSP70iV438K,J440A), HSP70iV442A,Y443V), and HSP70iE446V,R447A), as calculated by comparing band intensities for those observed using antibody SPA-811. Thus QPGVLIQVYEGER is part of the SPA-810 binding epitope. The resulting plasmids were introduced into COS7 cells to confirm functional protein expression. To identify HSP70i expression, monoclonal antibody SPA-810 generated against a 67-mer peptide partially overlapping with the peptide of interest, and polyclonal antibody SPA-811 which recognizes a C-terminal peptide within HSP70i downstream of a peptide of interest was used. Some expression of HSP70i was observed in COS7 cells as a consequence of the transfection process as indicated by SPA-810 and SPA-811 staining in lanes representing cells transfected with control DNA (vector), as represented in FIG. 6A. Of interest, antibody SPA-810 demonstrated reduced reactivity towards mutants HSP70iV438K,J440A), HSP70iV442A, Y443V), and HSP70iE446V, R447A), with resulting staining similar to that of endogenous HSP70i. However, there were no differences in reactivity to mutant HSP70i (Q435A). Together, these data demonstrate that the HSP70i sequence QPGVLIQVYEGER is part of an epitope recognized by SPA-810.

[0044] Depigmenting effects of mutant HSP70i were next investigated in vivo. Wild-type mice were gene gun vaccinated with wild-type or mutant HSP70i and/or TRP-2 encoding plasmids, and assessed for depigmentation. Results of these trials are represented in a graph of FIG. 6B. Image analysis indicated depigmentation is halted in mice receiving vaccinations with mutant versions of HSP70i and TRP-2 as compared to wild-type HSP70i. As represented in FIG. 6B, depigmentation was significantly decreased in the presence of variant sequences HSP70iV438K,J440A), HSP70iV442A,Y443V), and HSP70iE446V,R447A) as compared to unmutated HSP70i or HSP70iE446V,R447A). This indicates that a single amino acid difference in the target peptide region is sufficient to inactivate the depigmentation accelerating effects of HSP70i and supports the crucial involvement of the QPGVLIQVYEGER moiety in inducing autoimmune depigmentation.

[0045] Next, a trial was performed to determine whether HSP70i alone is sufficient to establish depigmentation in animals predisposed to developing autoimmune depigmentation, akin to human Vitiligo. Pmel-1 mice and wild-type mice were gene gun vaccinated with wild-type HSP70i and mutant HSP70i(Q435A) plasmids, as well as control DNA. The wild-type and the pml-1 mice were vaccinated three times every seven days with four μg of either wild-type (WT), HSP70i (Q435A) (MT), or control DNA (vector). Three months after the final vaccination, the pml-1 mice vaccinated with wild-type HSP70i displayed significantly increased ventral and dorsal depigmentation compared to mutant HSP70i or control DNA, as represented in FIG. 7. Graphs of FIG. 8 represent that upon quantification, the pml-1 mice receiving wild-type HSP70i-encoding DNA displayed significantly more depigmentation than either control DNA vaccinations and wild-type HSP70i, confirming that focal overexpression of HSP70i is sufficient to induce Vitiligo in disease-prone mice. As expected, no depigmentation was observed in the wild-type mice gene gun vaccinated with any plasmid. Importantly, ventral and dorsal depigmentation was significantly decreased in the pml-1 mice gene gun vaccinated with mutant HSP70i(Q435A) plasmid compared to the pml-1 mice vaccinated with control DNA. FIG. 10 is a scanned image of a wild-type mouse and a pml-1 mouse and their respective skin samples prior to testing. As shown in FIG. 10, the pml-1 mouse has already lost its differentiated melanocytes prior to testing. This indicates that a single missense nucleotide variation in HSP70i derivative peptide region is sufficient not only to abrogate the Vitiligo-inducing effect of natural HSP70i, but also to actively interfere with immune activation and subsequent depigmentation.

[0046] As the QPGVLIQVYEGER moiety proved crucial to the depigmentation process, its role in DC activation was further analyzed by FACS analysis. DC profiles were obtained from splenocytes of mice euthanized nine months after vaccination. The mouse splenocytes were stained for lymphocyte markers CD3, CD8, and IgM-Thy1.2, non-lymphocyte markers CD11b, CD11c, and F4/80, as well as DC maturation markers CD80, CD86, and MHC II. Stained cells were then followed by flow cytometry to delineate cell populations and their state of activation. For trials including pml-1 knockout mice, splenocytes were recovered twenty-five weeks after the final vaccination, and immediately stained. Initial gating was performed on live non-debris singlets, with subsequent gating towards CD11c versus CD11b cells using FACS canto equipment. Thy1.2, IgM, and CD3 positive cells were excluded from the final gating. Notably, marked differences were observed in the abundance of monocyte-derived subpopulations discriminated based on CD11b and CD11c expression levels, shown as R1 through R3 in graphs represented in FIG. 9A. Three distinct populations of cells were observed after gating for CD11b and CD11c cells in the non-lymphocyte population, with high (hi), low (lo) or intermediate (int) levels of expression. Macrophages are observed in the R1 population as CD11bint/cD11cintlo, while DCs are amongst the R2 and R3 populations as CD11binfCD11cint and CD11biloC11chi respectively. The R3 conventional DC population remained unchanged, yet monocyte derivative populations were markedly biased towards CD11b expression within mice vaccinated with DNA encod-
ing mutant HSP70i when compared to wild-type HSP70i encoding or control DNA. Graphs in FIG. 9B represent quantification of CD11b and CD11c expressing cells determined differential expression of these markers among leukocytes after vaccination with wild-type or mutant HSP70i(Q435A) plasmids. Intriguingly, the population R1 was increased by the mutant HSP70i(Q435A) vaccination, whereas the same population is suppressed after vaccination with wild-type HSP70i. The DC population R2 (CD11b/CD11cint) representative of pro-inflammatory DC's shows an opposite trend. These data demonstrate that HSP70i can drive the relative abundance of immune cell subpopulations associated with autoimmune depigmentation. The QPGVLIQVYEG moiety is involved in depigmentation by defining the prevalence of dendritic cell subsets important for CTL activation, and mutations in this region alter the macrophage and DC ratios.

[0047] After determining that mutant HSP70i can halt disease in Vitiligo-prone mice, trials were performed to investigate potential side effects. Specifically, when inhibiting autoimmune, there is also a concern of inhibiting immunity in general and patients may not be able to mount an anti-tumor response. Therefore, wild-type and CD8 knockout mice were gene gun vaccinated first with autoimmune treatments as described above and then gene gun vaccinated with tumors.

[0048] Wild-type and CD8 KO mice were vaccinated with control DNA and TRP-1, Wild-type HSP70i and TRP-1, or mutant HSP70i(Q435A) and TRP-1. The wild-type and CD8 KO mice were given a dosage of three μg of each plasmid for a total of six μg each and were vaccinated five times, six days apart. FIGS. B1 and B2 are images showing the wild-type and CD8 KO mice six weeks after final vaccination when they were assessed for depigmentation. Thereafter, the wild-type and CD8 KO mice were injected with two booster vaccinations of 100,000 B16F12 cells, five days apart. FIGS. 10 and 11 are graphs representing the observed tumor growth in the wild-type and CD8 KO mice.

[0049] To determine DC stimulation within the wild-type and CD8 KO mice, monocyttes were harvested and magnetically sorted from peripheral blood. 250,000 cells were added to five milliliters of media (RPMI, 10% FBS, Pen/strep) in teflon containers. GM-CSF and IL–4 were added to drive DC differentiation and half of the media was changed every other day. On day 6, one μg/mL of either His tag sorted wild-type or mutant HSP70i(Q435A), commercially available HSP70i, LPS, or nothing was added. On day 7, the cells were stained for CD11c, CD80, CD86, CD83, HLA-DR, and/or HLA-DR all directly with conjugated fluorochromes. FACS analysis gating on DC population was performed (measured by forward and side scatter, and CD11c positivity). The results of the trial are represented in graphs of FIGS. 12, 13, 14, and 15. These results reveal that animals treated with mutant HSP70i are still protected from tumors compared to animals treated with wild-type HSP70i.

[0050] More recently, trials have been performed to understand the differential effects of wildtype HSP70i and mutant HSP70i on DC activation by studying enhanced expression of activation markers in response to either isoform. It is believed that the natural variant activates DC in vitro while the mutant HSP70i does not.

[0051] In the above trials, the exclusive, causative role of HSP70i in inducing autoimmune depigmentation in mice was examined. Using constitutive and inducible HSP70 knockout mouse models, it was determined that the inducible, but not constitutive isoform of HSP70i is solely responsible for inducing and perpetuating an autoimmune response.

[0052] Previous vaccination studies using HSP70i in combination with melanocyte antigens at suboptimal levels have shown that immune responses can be elicited towards cells bearing these target antigens. This specificity is best attributed to the chaperone function of HSP70i in binding these antigens. In addition, these trials showed that depigmentation preferentially occurred after inclusion of HSP70i in the vaccine, demonstrating the importance of this protein in perpetuating and precipitating autoimmune responses.

[0053] Importantly, these trials show that injecting mice with a combination of HSP70i and melanocyte antigen encoding plasmids is sufficient for inducing Vitiligo, and this response is mediated by CD8+ T cells. These trials demonstrate the shared relationship between Vitiligo and melanoma in the targeting of specific cells mediated by HSPs. Here, it was determined that depigmentation in wild-type mice occurred with and distal to the site of the gene gun vaccination, as represented in FIGS. 1, 2, 3A, 3B, 7, and 8. Cytotoxicity towards melanocytes is now believed to be due to an antigen-specific and systemic immune response. Here, the HSP70i KO mice displayed reduced killing of melanocyte antigen pulsed splenocytes, indicating a role for HSP70i in the immunogenicity towards these cells.

[0054] In the case of anti-tumor immunity, HSP70i is believed to efficiently chaperone tumor antigens, drive DC activation, or directly activate the adaptive immune system. In the latter, APCs will be activated leading to the recruitment of effector cells, and the eventual killing of target cells. Interestingly, in the above trials both in the wild-type and HSP70i KO mice that were vaccinated with TRP-1 plasmid, killing of TRP-2 pulsed splenocytes was observed indicating that epitope spreading had occurred.

[0055] Previous studies have observed that perilesional skin of expanding lesions in Vitiligo patients contains greater numbers of T cell infiltrates as compared to control skin. T cell mediated autoimmune responses to melanocytes are accompanied by the presence of melanocyte specific CTLs in Vitiligo patient skin. The histological staining of the above trials show that the skin from the wild-type mice had greater numbers of T cells as compared to the HSP70-1 KO mice after vaccinating each with TRP-1, and there was an even greater increase with the addition of HSP70i plasmid (not shown). These observations were inversely related to the loss of melanocytes within the wild-type mice, which can be attributed to increases in self-reactive CTL. Generally, vaccinating with TRP-1 (Tyrp1ee) elicits highly effective T cell responses; however, it was shown that in the absence of endogenous HSP70i, T cell tolerance towards melanocytes is maintained. On the other hand, removing constitutive HSP70i still resulted in melanocyte death. This confirms that only HSP70i is required to elicit an autoimmune response.

[0056] Extracellular HSP70i has been shown in previous studies to activate DC directly, and via the peptides they chaperone. These studies have shown that the C-terminal portion of HSP70i (residues 359-610) stimulates DC, and supports Th1 polarization, driving cytokine production of TNF-α, IL–12, and release of NO release. Therefore, a peptide sequence QPGVLIQVYEG (HSP70i434-444) was identified as a potential target for blocking entities to prevent DC activation. From the trials above, the peptide sequence QPGV-
LIQVYEG was implicated as a novel target in preventing an autoimmune response, and a potential therapy in autoimmune disorders such as Vitiligo.

[0057] The stand-alone function of HSP70i was evaluated in a spontaneous mouse model of Vitiligo to demonstrate its immune enhancing effects. The pemel-1 mice were engineered to have self reactive T cells expressing a T cell receptor reactive to the melanocyte antigen gp100. These pemel-1 mice faithfully mimic human Vitiligo in which melanocyte reactive T cells escape clonal deletion, and stress is shown to augment the T cell response. The pemel-1 mice were vaccinated not only with wild-type HSP70i to look for accelerated establishment of autoimmune disease, but also with a representative mutant sequence to look for abrogation of the same response. Comparison of Fig. 7 and 16 show that the mutant HSP70i may be used to reduce the progression of Vitiligo but to regain pigmentation of the white skin.

[0058] Importantly, it had already been shown that as little as a single residue change in this region is sufficient to fully prevent the depigmentation inducing effects in mice when vaccinated in combination with TRP-2. Vaccination with mutant HSP70i(Q435A) fully abrogated the gradual depigmentation observed in aging mice or animals vaccinated with the plasmid control DNA. The trials showed that depigmentation is accelerated after vaccination with wild-type HSP70i, again demonstrating the importance of this protein in precipitating disease.

[0059] Here, FACS analysis of the pemel-1 mice splenocytes revealed phenotypes of CD11c and CD11b cells as reported in the wild-type mice. Previous studies reported that Treg homing to skin is severely reduced in Vitiligo patients, which may in part be associated with a decline of the skin homing chemokine CCL22. Importantly, the above trials demonstrate that the same subset of Treg inducing macrophages is down or upregulated by wild-type and mutant HSP70i(Q435A) respectively. Thus, wild-type HSP70i may contribute to Vitiligo through the inhibition of Treg via reduced macrophage activity, and through supporting Th17 mediated autoimmunity by inflammatory DC. It is believed then that HSP70i may be acting on two fronts, by activating CTLs via DC activation, and simultaneously downregulating Treg activity. By contrast, HSP70i(Q435A) drives an opposite, anti-Vitiligo phenotype. The differential immune effect of wild-type versus mutant HSP70i are believed to be related to mutations in the DC activating peptide PGPVLIQYEG.

[0060] HSP70 has been implicated in previous studies in various autoimmune disorders including diabetes and rheumatoid arthritis. In addition, the release of HSP70i by necrotic cells may augment inflammation such as that seen with acute myocardial infarction (AMI). This renders therapeutic measures that interfere with immune functions of HSP70 of primary importance. Here, the trials showed that HSP70i plays a crucial role in another autoimmune disorder, Vitiligo. The data indicate that HSP70i has an important role in inducing depigmentation by activating DC and subsequent breaking of T cell tolerance to self antigens. This implicates HSP70i as a crucial molecule defining a bottleneck in the etiopathology of Vitiligo.

[0061] Preventing secreted HSP70i from inducing an autoimmune response will prove as an important strategy for treating autoimmune disorders. Currently, treatments for Vitiligo typically involve local immune suppression through topical hydrocortisone application, and psoralen plus UVA (PUVA) therapy; however, the success rate is quite limited and involves long lag periods. Several groups have implicated the local production of reactive oxygen species (ROS) in Vitiligo as well as other autoimmune disorders. Therefore, it has been proposed in the art to increase catalase activity to protect melanocytes; however, this will similarly support T cell longevity. Thus there is a dire need for treatment measures that can inhibit and preferentially invert depigmentation. The trials above show that the peptide region QPGVLIQYEG is crucial for this response. The trials not only implicate HSP70i in Vitiligo, but provide a mechanism by which the autoimmune response is activated and maintained. In knockout animals, depigmentation could not be established with or without wild-type, or mutant HSP70i. Therefore, it is now believed that depigmentation is fully dependent on CD8 cytotoxic T cells. Using HSP70i expressing variants to the DC binding peptide PGPVLIQYEG in vaccinations, therapeutic measures can be established to reduce or prevent autoimmune diseases including Vitiligo, for which effective measures do not exist to date.

[0062] In view of the above, a natural, human HSP70i derived peptide that is believed to be pivotal to depigmentation has the sequence: QPGVLIQYEG. The mutant for which autoimmune-inhibiting properties were investigated is APGVLIQYEG. Both depigmentation-enhancing and depigmentation-inhibiting properties are seen with the encoding sequence incorporated into a plasmid containing the full HSP70i cDNA sequence.

[0063] Although the invention has been described in terms of the peptide comprising the sequence APGVLIQYEG, other peptides derived from the inducible heat shock protein 70 may be used for treating autoimmune diseases and are therefore within the scope of this invention. These autoimmune-inhibiting peptides are characterized by their ability to reduce or prevent activation of DCs due to HSP70i. Such autoimmune-inhibiting peptides are preferably adapted to a vaccine comprising the peptide derived from HSP70i and a plasmid containing the full HSP70i DNA sequence encoding the peptide. The resulting vaccine may be combined with a vaccine administration device to form a vaccination kit. An example of the administration device is, but should not be limited to, a gene gun.

[0064] According to a preferred aspect of the invention, human patients susceptible to or suffering from autoimmune diseases can be treated using vaccines formed from the autoimmune-inhibiting peptides. In the case of Vitiligo, it is believed that the patients will experience reduced depigmentation in their skin once vaccinated with the vaccine as described above.

{The figures in Attachment A labeled Pmel and B6 Vitiligo figures A, B, C, and D do not have descriptions. If this application would benefit from their inclusion, please supply a short description of the figures so they may be added.}

[0065] While the invention has been described in terms of specific embodiments, it is apparent that other forms could be adopted by one skilled in the art. For example, the specific peptide derived from the HSP70i could differ from that shown, and materials and processes other than those noted could be used. Therefore, the scope of the invention is to be limited only by the following claims.
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1. A vaccine for treating an autoimmune disease comprising a peptide derived from inducible heat shock protein 70, the peptide encoded in a plasmid containing a full inducible heat shock protein 70 DNA sequence.

2. The vaccine of claim 1, wherein the peptide is characterized by its ability to reduce activation of dendritic cells.

3. The vaccine of claim 1, wherein the peptide is an inducible heat shock protein 70 expressing variant to the dendritic cell binding peptide with the sequence QPGVLIQVYEG.

4. The vaccine of claim 1, wherein the peptide has the sequence APGVLIQVYEG.

5. The vaccine of claim 1, wherein the peptide is derived from human inducible heat shock protein 70.

6. The vaccine of claim 1, wherein the autoimmune disease is Vitiligo and the vaccine is effective to reduce depigmentation in the skin of a human suffering from Vitiligo when administered to the human.

7. A vaccination kit comprising a vaccine administration device and the vaccine of claim 1.

8. The vaccination kit of claim 7, wherein the administration device is a gene gun.

9. A method of treating an autoimmune disease comprising administering to a human patient the vaccine of claim 1.

10. The method of to claim 9, wherein the patient is currently suffering from an autoimmune disease.

11. The method of to claim 9, wherein the patient is genetically prone to an autoimmune disease.

12. A method of treating an autoimmune disease, the method comprising:
- deriving a peptide from inducible heat shock protein 70;
- forming a vaccine comprising the peptide derived from inducible heat shock protein 70, the peptide encoded in a plasmid containing a full inducible heat shock protein 70 DNA sequence; and then
- vaccinating a human patient with the vaccine.

13. The method of claim 12, wherein the peptide is characterized by its ability to reduce activation of dendritic cells.

14. The method of claim 12, wherein the peptide is an inducible heat shock protein 70 expressing variant to the dendritic cell binding peptide QPGVLIQVYEG.

15. The method of claim 12, wherein the peptide is APGVLIQVYEG.

16. The method of claim 12, wherein the peptide is derived from human inducible heat shock protein 70.

17. The method of claim 12, wherein the autoimmune disease is Vitiligo and the vaccine is effective to reduce depigmentation in the skin of a human suffering from Vitiligo when administered to the human.

18. The method of claim 12, wherein the administration device is a gene gun.

19. The method of to claim 12, wherein the human patient is currently suffering from an autoimmune disease.

20. The method according to claim 12, wherein the human patient is genetically prone to an autoimmune disease.