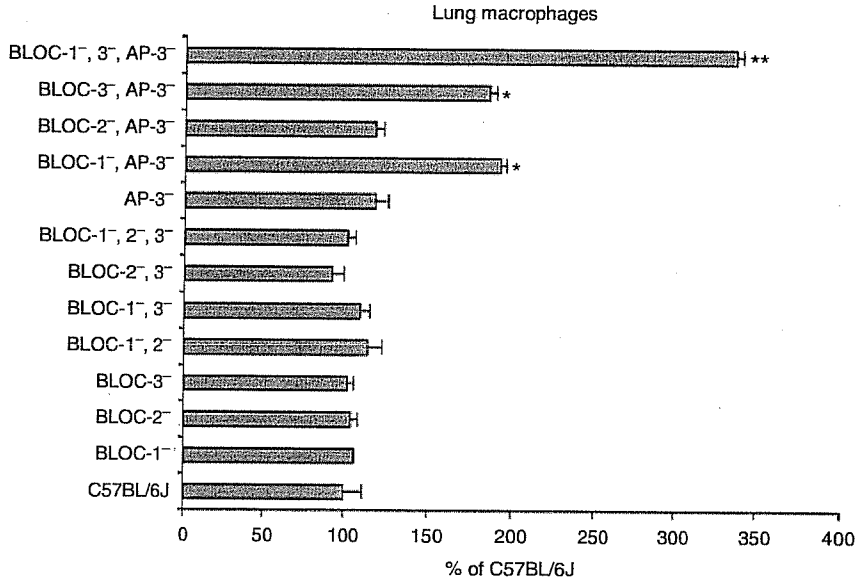


**Figure 7: Macrophage accumulation in lungs of multiple HPS mutants.** The macrophage lysosomal enzyme N- acetyl-β-glucosaminidase was assayed in lung homogenates of multiple HPS mutants by p-nitrophenol-conjugated N-acetyl- β-D-glucosaminide substrate and expressed relative to lung N- acetyl-β-glucosaminidase concentrations of C57BL/6J. $n = 4$, *$p < 0.01$, **$p < 0.001$.

protein complex associate into smaller attenuated complexes which retain some residual functional activity.

If mutations in recessive double mutant mice are in a common pathway, phenotypes will resemble one or the other parent. If the mutations are in independent pathways, a new, often more severe, phenotype usually appears because of additive or interactive effects. These studies establish that interactions between HPS protein complexes in the biosynthesis of LROs are widespread and that this interaction is organelle and tissue specific. Analysis of granule secretion from CTL of a wide variety of single HPS mutants likewise confirms that their phenotypes are organelle and tissue specific (33). This likely reflects differences in the protein machinery for biogenesis and/or secretion of LROs.

### *Viability/robustness of double/triple mutants*

The double and triple mutant HPS mice uniquely provide a measure of the role(s) of HPS protein complexes in general health and physiology in mammals. A major conclusion of our studies is that the BLOC complexes are not required for viability. All homozygous double mutants involving all combinations of the three HPS BLOC complexes are viable, and these young mutants are apparently healthy. Also, all produce normal litter sizes. Moreover, the homozygous triple mutant BLOC-1⁻, 2⁻, 3⁻ is viable, attains midlife (nine months) with no apparent health problems and produces normal litter sizes despite being deficient in all BLOC complexes. There are at least two explanations for the surprising robustness of multiple BLOC mutants. The first is that redundant systems for BLOC action in critical biological functions may exist, at

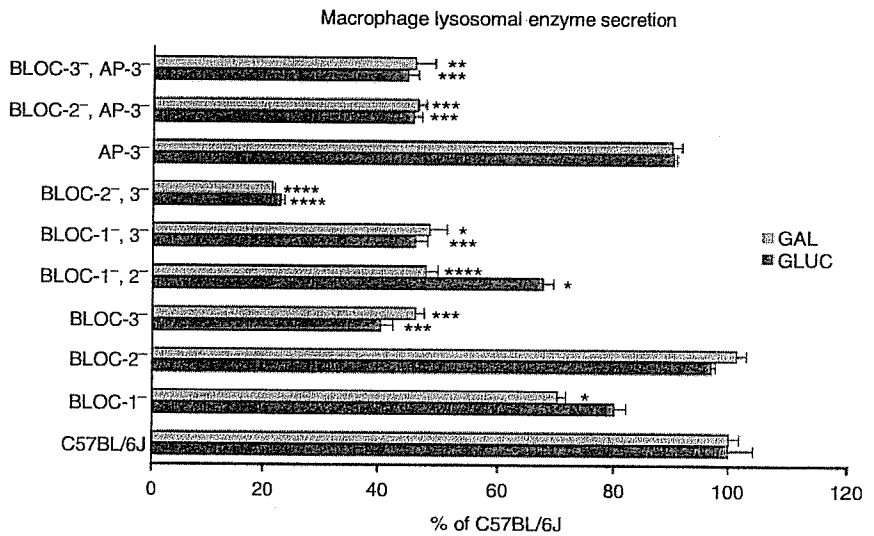


**Figure 8: Lysosomal enzyme secretion from macrophages of multiple HPS mutants.** The secretion of β-glucuronidase (GLUC) and β-galactosidase (GAL) was measured following treatment of macrophages with 50 mM $NH_4Cl$. The values are expressed as a percentage of lysosomal enzyme secretion from macrophages of C57BL/6J. Possible interaction between BLOC-1 and AP-3 was not tested due to an inability to breed adequate numbers of the BLOC-1⁻, AP-3⁻ double mutant. $n = 3$, *$p < 0.05$, **$p < 0.02$, ***$p < 0.01$, ****$p < 0.001$.

least in early life. Second, many HPS genes are found only in higher eukaryotes (3). This strongly implies that they have evolved to enable the biosynthesis of specialized LROs rather than to provide functions critical for viability. They affect pigmentation, bleeding time and lung surfactant recycling which are important for quality of life but are not necessarily required for viability. The importance of HPS genes does become apparent in later life. For example, it has been demonstrated that BLOC functions are critical for long-term (>1 year) robustness and survival (not analyzed here) (16) of several HPS mouse mutants. Life spans are reduced in pallid (BLOC-1$^-$) and cocoa (BLOC-2$^-$) single mutants and are severely reduced in ruby eye/pale ear (BLOC-2$^-$, 3$^-$) double mutants. Likewise, lung disease, the most severe clinical consequence of HPS, typically is not apparent until the fourth decade of life in HPS patients (5). Finally it should be noted that the relative viability and robustness of all multiple BLOC mutants observed here may be enhanced by maintenance in relatively pristine laboratory conditions.

In contrast to BLOC multiple mutants, large reductions in viability and breeding ability were noted whenever the AP-3 mutation was combined with any BLOC in multiple mutants. This occurred despite the fact that normal litter sizes were produced by all single mutants involving either AP-3 or the particular BLOC. The drastic effects of AP-3 mutations on viability were especially apparent in the case of the BLOC-1$^-$, 2$^-$, 3$^-$, AP-3$^-$ mutant, where only two homozygous quadruple mutants were produced despite the Mendelian prediction of 21 quadruple mutants. The probability of this result occurring by chance is extremely low ($p < 10^{-8}$ by the binomial distribution test) indicating considerable loss in viability of this genotype. The fact that low litter size was not specifically a defect in either the male or female multiple mutant parent suggests a block in postfertilization development or birthing. Thus AP-3 and BLOC genes interact in some aspect of fertility. Loss of AP-3 complex function in CTL-mediated immunity (34) and in CD1d-mediated lipid antigen presentation (35,36) may contribute.

***Pigmentation effects in double/triple mutants***

Epistatic effects of murine HPS genes on pigmentation are more complex than those on viability. It has long been recognized that mutations within the BLOC-1 complex produce the most pronounced hypopigmentation effects of any HPS mutation. We observed no further diminution of pigmentation when the BLOC-1$^-$ mutation was combined in double or triple mutants with any other BLOC mutation or combination of mutations. This is consistent with action of the BLOC-1 complex upstream of the other BLOCs. A similar conclusion was derived from the analysis of melanosome morphology and coat color in a wide variety of murine mutants (37,38) and from analysis of the coat color of BLOC-1$^-$, 3$^-$ double mutant mice (39). However, mild interaction was noted between BLOC 2 and 3 genes as the BLOC-2$^-$, 3$^-$ double mutant exhibited hypopigmentation and decreased density of the melanosomes of the RPE relative to that of either single mutant. This result suggests that the three BLOCs do not interact solely in a simple linear pathway in controlling pigmentation. Likewise, and similar to the observed effects on viability, all multiple mutants involving BLOCs and AP-3, including those involving BLOC-1, resulted in a more pronounced hypopigmentation than the corresponding single mutants, consistent again with AP-3 and BLOCs operating in separate interacting or parallel pathways. The results of these studies are consistent with demonstrations that the BLOC-3 (pale ear) and AP-3 (pearl) gene products interact indirectly and independently to produce more severe melanosome, lysosome and platelet dense granule phenotypes (18). Analyses of vesicle trafficking at the cellular level have likewise concluded that the AP-3 and BLOC complexes exert differential control over vesicle trafficking. For example, loss of AP-3 function results in abnormal trafficking of lysosomal membrane proteins to the plasma membrane (40,41), a defect not observed in any BLOC mutant. Nevertheless it remains possible that AP-3 and the various BLOCs may also act within a linear pathway as proposed by Richmond *et al.* (42). Under such a scenario, a mutation in AP-3 would act upstream of BLOC-3 to sequester melanosomal proteins within multivesicular bodies. This in turn could prevent (as observed within the choroid of the BLOC-3$^-$, AP-3$^-$ double mutant) the downstream accumulation of giant melanosomes normally associated with the BLOC-3 deficiency. A similar scenario [i.e. BLOC-2 acting upstream of BLOC-3 (42)] might explain the less dense RPE melanosomes observed in BLOC-2$^-$, 3$^-$ double mutant.

Chemical analyses of hair melanin substantiated visual coat observations. The lower levels of pheomelanin in hair of double and triple BLOC mutants are consistent with evidence that the ratio of pheomelanin to eumelanin is increased in those cases where tyrosinase activity is decreased (43). Tyrosinase function is likely compromised in many, if not all HPS mutants. For example, tyrosinase is missorted in melanocytes of HPS2 patients (44) and is localized to aberrant macroautophagosomes in HPS1 melanocytes (45).

In most cases where epistatic effects were observed on coat color, corresponding enhanced hypopigmentation was observed by visual examination of eye pigmentation. An exception was that no further loss of pigment was apparent in those cases where AP-3 was combined in multiple mutants with BLOC-1$^-$. However, closer analysis by the very sensitive electron microscopic approach revealed novel quantitive and/or qualitative effects on melanosomes of the RPE and/or choroid in all multiple mutants resulting in significant alterations to melanosome morphology and number. This indicates interactions of all HPS genes in the formation of eye melanosomes, especially within the choroid. Differences between the RPE and choroid may reflect their differing embryological

origin, with the former derived from the optic cup and the latter from the choroid. These results emphasize the importance of the use of sensitive techniques in the detection of epistatic interactions. They likewise indicate that the BLOC and AP-3 complexes do not act solely in a simple linear pathway; rather they interact in a complex manner to effect melanosome biogenesis. It should be noted that the relative sensitivity of particular assays may limit interpretation of heterogeneity in phenotypes of LROs in various tissues of multiple mutants.

### *Lung abnormalities in double/triple HPS mutants*

We examined epistatic effects of HPS genes on lung structure and composition because fibrotic lung disease is an especially pressing clinical problem among HPS patients, especially *HPS1* and *HPS4* (46), and an increasing body of evidence (17,21,47) implicates an LRO, the lamellar body, in HPS lung disease. Also, our previous studies (17) showed that, while single murine HPS mutants exhibit relatively small lung abnormalities, one double mutant, ep/pe, had significant lung disease. This suggested in turn that other double/triple mouse HPS mutants might prove useful as animal models of HPS lung disease.

Indeed, significant lung abnormalities were observed in several double/triple mutants, including all double and triple combinations of any BLOC with AP-3 and in two multiple mutants among BLOCs (BLOC $1^-$, $3^-$ and BLOC $1^-$, $2^-$, $3^-$). A distinguishing feature of the lung abnormality in all the double/triple HPS mutants is the presence of giant surfactant B-positive lamellar bodies in type II cells, emphasizing that all mutant genes likely affect lung function through their effects on this specialized and critical lung LRO. All enlarged granules were positive for surfactant B, confirming their lamellar body origin. The presence of giant lamellar bodies would be expected to increase lung lipid surfactant levels. Indeed, lung p-lipid levels were elevated only in the lungs of those multiple mutants exhibiting giant lamellar bodies. Thus, HPS genes established as critical in the synthesis of LROs of other tissues are likewise critical for the synthesis of normal lamellar bodies of type II cells.

All the various double/triple HPS mutants are potentially useful animal models for lung disease in human HPS. The previously characterized ep/pe double mutant (17) replicates lung disease in human HPS (47,48) in that both exhibit significant numbers of infiltrating cells, a majority of which are foamy macrophages. The lack of infiltrating macrophages (despite enlarged lamellar bodies) in two multiple HPS mutants not involving AP-3 (BLOC-$1^-$, $3^-$ and BLOC-$1^-$, $2^-$, $3^-$) stands in contrast to multiple HPS mutants involving AP-3. Macrophages accumulated in three out of four of the latter. These results are consistent with our findings of strong interaction of AP-3 with all BLOC complexes in the biosynthesis of all LROs. A useful area of future studies will be to determine if the progression to overt lung disease differs in multiple mutants containing and lacking macrophage infiltration. Clearly, progression to overt emphysema occurs relatively rapidly in the BLOC-$3^-$, AP-$3^-$ double mutant (17). As previously noted (17), an unexplained difference between human HPS lung disease and lung disease in mouse HPS models is that the former presents largely as fibrosis while the latter presents as emphysema. This may be an inherent species difference. Alternatively it may reflect the relatively pristine murine housing conditions, which would reduce infections and other lung insults.

### *Secretion of lysosomal enzymes*

Our analyses of lysosomal enzyme secretion revealed a severity hierarchy among the single HPS mutants quite different from their effects on pigmentation. The most severe phenotype occurred in the BLOC-$3^-$ (pale ear) mutant, which has relatively mild effects on pigmentation or on other HPS phenotypes such as numbers of platelet dense granules (24). Severe effects of BLOC-3 deficiencies on lysosomal enzyme secretion are not limited to macrophages. Abnormal secretion of lysosomal enzymes from thrombin-treated platelets (elevated) and from testosterone-treated kidney proximal tubule cells (depressed) has been documented (24).

### *General conclusions*

Somewhat surprisingly, a profound deficiency of BLOC components in multiple mutants is not fatal, though additional studies are needed to delineate the effects of these losses on long-term robustness. These *in vivo* studies have revealed that interactions (Figure 9) among BLOC and AP-3 complexes in the synthesis of LROs are more extensive than indicated by common protein interaction analyses. These interactions are tissue and cell specific, likely reflecting the formation of cell specific physiological modules (49) that interact to form specialized LROs in mammals. Other profound differences in cell and tissue phenotypes of the various mouse HPS mutants have been documented (3,22,31). These tissue specific interactions are critical for understanding the biology of HPS proteins and likely will prove important for the design of therapies for this presently untreatable disease.

## Materials and Methods

### *Breeding of double, triple and quadruple mutants*

To produce double mutants, single mutants homozygous at two different HPS loci were mated to produce F1 progeny, which were mated to produce an F2 generation. Double mutants (1 of 16 progeny expected) were provisionally selected from F2 progeny by the appearance of a new lighter coat or eye phenotype. In some cases, F2 progeny with a phenotype identical to either homozygous single mutant and identified as heterozygous at the other HPS locus by molecular genotyping (3) were mated to produce double mutants (expected frequency = 1/4). Double mutant stocks were maintained by self-mating, or, if low breeding efficiencies were observed, by mating double homozygotes to mice homozygous for one mutant gene and heterozygous for the other.

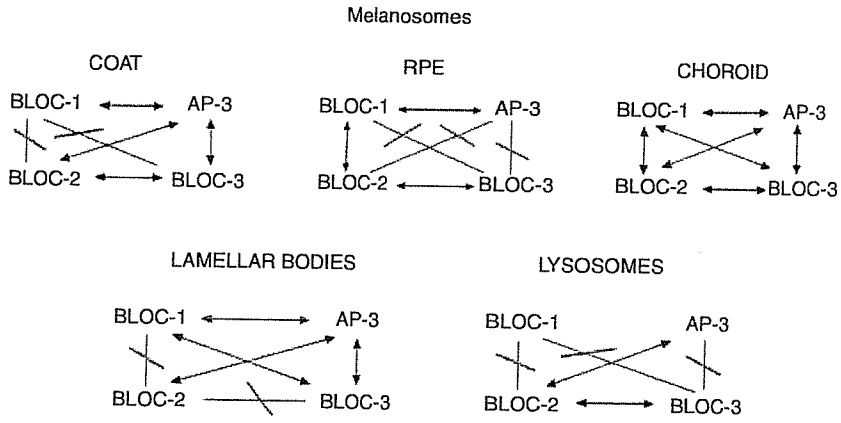


**Figure 9: *In vivo* interactions between HPS protein complexes in the functions of three LROs.** Interactions detected between BLOC genes or between BLOC and AP-3 genes are depicted with a double arrowhead line. Oblique lines indicate that no interaction was observed. Possible interaction between BLOC-1 and AP-3 was not tested due to an inability to breed adequate numbers of the BLOC-1$^-$, AP-3$^-$ double mutant. RPE (retinal pigment epithelium).

Triple mutants were produced in a similar fashion. Mice doubly mutant in two HPS genes were mated to other mice doubly homozygous for mutations in one common and one different HPS gene, to produce F1 and F2 generations as described. Offspring with homozygous mutations at all three HPS loci were selected by molecular genotyping (expected frequency = 1/4). Alternatively, mice doubly mutant in two HPS genes were mated to mice homozygous for a mutation in a third HPS gene to produce an F1 generation. F1 mice were mated to mice doubly mutant in the original two HPS genes. F2 progeny identified as homozygous for the original two HPS genes and heterozygous for the third gene were mated to produce triply homozygous mutants (expected frequency = 1/4).

To produce quadruple mutant mice (deficient in the 3 BLOC complexes and AP-3), $Hps1^{ep}/Hps1^{ep}$, $Pldn^{pa}/Pldn^{pa}$, $Hps5^{ru2\text{-}J}/Hps5^{ru2\text{-}J}$ mice were mated with $Hps1^{ep}/Hps1^{ep}$, $Pldn^{pa}/Pldn^{pa}$, $Ap3b1^{pe}/Ap3b1^{pe}$ mice to produce $Hps1^{ep}/Hps1^{ep}$, $Pldn^{pa}/Pldn^{pa}$, $Ap3b1^{pe}/+$, $Hps5^{ru2\text{-}J}/+$. These were mated to $Hps1^{ep}/Hps1^{ep}$, $Pldn^{pa}/Pldn^{pa}$, $Hps5^{ru2\text{-}J}/Hps5^{ru2\text{-}J}$. All progeny were molecularly typed to identify $Hps1^{ep}/Hps1^{ep}$, $Pldn^{pa}/Pldn^{pa}$, $Hps5^{ru2\text{-}J}/Hps5^{ru2\text{-}J}$, $Ap3b1^{pe}/+$ mice. These were mated to produce the $Hps1^{ep}/Hps1^{ep}$, $Pldn^{pa}/Pldn^{pa}$, $Hps5^{ru2\text{-}J}/Hps5^{ru2\text{-}J}$, $Ap3b1^{pe}/Ap3b1^{pe}$ quadruple mutant.

The genetic identity of all double, triple and quadruple mutants analyzed in this report was confirmed by molecular procedures (3) which verified that all mutants contained appropriate homozygous mutations at each gene.

### *Isolation of mouse tissues*

Tissues were isolated from 6 to 10 week old mice, washed with phosphate buffered saline and stored at −70 °C. Tissues were thawed and homogenized with a Polytron homogenizer in 0.25 M sucrose/0.02 M imidazole, pH 7.2.

### *Chemical analyses of melanin*

Eumelanin and pheomelanin were quantitatively analyzed (19,20) by high-performance liquid chromatography (HPLC) based on the formation of pyrrole-2,3,5-tricarboxylic acid (PTCA) by permanganate oxidation of eumelanin and 4-amino-3-hydroxyphenylalanine (4-AHP) by hydriodic acid reductive hydrolysis of phemelanin, respectively. These specific degradation products were determined by HPLC.

### *Phospholipid assay*

Lungs were extracted with chloroform-methanol, and the lipid phase used for analysis of inorganic phosphate by the Fiske–SubbaRow reagent (17).

### *Lysosomal enzyme secretion from macrophages*

Peritoneal macrophages collected four days after thioglycollate injection were washed, suspended in DMEM with 10% FBS, counted and plated overnight on glass coverslips (25). The coverslips were washed three times with PBS and treated with DMEM (control) and with DMEM + 50 mM $NH_4Cl$ (freshly prepared). The plates were incubated for 4 h at 37 °C. Medium and cells were collected separately and assayed for the lysosomal enzymes β-glucuronidase and β-galactosidase.

### *Histology*

Lungs were inflation-fixed at 20 cm $H_2O$ pressure with 10% buffered formalin, embedded in paraffin and stained with hematoxylin and eosin (17). For immunohistochemistry, slides were deparaffinized with a graded series of alcohols, and antigen was exposed with proteinase K treatment (17). Slides were processed for antibody staining using rabbit anti-sheep polyclonal antibody to surfactant protein B (Chemicon International, Temecula, CA, USA) at 1 : 500 dilution as described (17).

### *Electron microscopy*

Eyes were fixed in glutaraldehyde, postfixed in osmium tetroxide and embedded in spur resin before viewing on a Siemens 101 Electron microscope at an accelerating voltage of 80 kBv (32).

### *Lysosomal enzyme assays*

Thioglycollate-elicited macrophages were isolated from the peritoneal cavity of mice four days after intraperitoneal injection of 4% Brewers thioglycollate Broth (Difco Microbiology, Kansas City, MO, USA), and cultured overnight in medium 199 containing 10% newborn calf serum on plastic coverslips prior to treatment with 50 mM $NH_4Cl$ (50). β-glucuronidase and β-galactosidase in tissues and macrophages were assayed with fluorescent methylumbelliferyl substrates (51).

N- Acetyl-β – glucosaminidase was assayed spectrophotometrically (23). Lung homogenates were mixed with 10 µL of 0.1% Triton X-100 in a microtitre plate. Twenty microliters of p-nitrophenyl N-acetyl- β –D-glucosaminide (Sigma, St Louis, MO, USA) was used as a substrate, and the plate (50 µL/well) was incubated at 37 °C for 15 min. The reaction was stopped by the addition of 200 µL of 0.2 M sodium carbonate and absorbance was measured at 405 nm.

Protein was determined with the Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA, USA). Statistical comparisons utilized the Student's *t*-test and the binomical distribution test.

## Acknowledgments

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# Extracellular Heat Shock Proteins (HSP) in Immune Response: A Guide for Cross-Presentation

Yasuaki Tamura and Noriyuki Sato

Department of Pathology
Sapporo Medical University School of Medicine
Sapporo, Japan

**Running Title:** HSP in immune response

**Abbreviations:** APC, antigen presenting cells; CTL, cytotoxic T lymphocytes; ER, endoplasmic reticulum; HLA, human leukocyte antigen; HSP, heat shock proteins; MHC, major histocompatibility complex; OVA, ovalbumin; TAP, transporter-associated antigen processing; TCR, T cell receptor; TLR, Toll-like receptor.

**Correspondence/Reprint request:**

Dr. Yasuaki Tamura
Department of Pathology
Sapporo Medical University School of Medicine
South 1, West 16, Chuo-ku, Sapporo 060-8556, Japan.
Phone +81-11-611-2111 (Ext. 2691)
Fax: +81-11-642-2311
E-mail: ytamura@sapmed.ac.jp

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## Abstract

## Introduction

Priming of CD8+ T cells requires recognition through the T cell receptor (TCR) of peptide-major histocompatibility complex (MHC) class I molecules on the surfaces of appropriate antigen presenting cells (APC). In most cell types, MHC class I-associated peptides are derived solely from protein synthesized within the cell. However, bone marrow-derived APC can directly endocytose proteins into the MHC class I presentation pathway, a process known as cross-presentation, and play an important role in priming antigen-specific naïve CD8+ T cell responses to tumor cells, virus-infected cells and transplants that cannot access the classical pathway for MHC class I presentation. Although several different bone marrow-derived cells can cross-present antigens in vitro, the main APC able to do so in vivo are dendritic cells (DC). However, to enter the cross-presentation pathway, it is evident that certain forms of exogenous antigens can be favorable candidates. A large spectrum of exogenous antigens such as apoptotic tumor cells, tumor cell exosomes, immune complexes, virus-like proteins and heat shock proteins (HSP) has been shown to elicit cytotoxic T lymphocyte (CTL) responses through cross-presentation. Among them, HSP have a unique feature because of specific receptors expressed on the DC.

HSP are abundant primordial molecules expressed in all cells. Since 1984, various publications have shown that immunization of mice, rats, and frogs with purified preparations of selected HSP isolated from cancers leads to protective immunity against the cancer used as the source of the HSP [1]. The basis of the tumor-specific immunogenicity of these molecules lies not in the molecules themselves but in the array of peptides, including antigenic peptides chaperoned by them. These experiments and the ideas derived from them form the basis of an approach to immunotherapy for human cancer that began in 1995 and the feasibility of its use to treat metastatic melanoma and colon cancer patients has been demonstrated [2-4].

Recent reports have demonstrated that administration of exogenous Hsp70 and gp96 induces cross-presentation by DC [5]. In addition, specific receptors such as LOX-1 and CD91, for Hsp70 and gp96 have been demonstrated. On the other hand, Hsp90 is one of the most abundant cytosolic proteins and it has been demonstrated that Hsp90 isolated from tumor cells elicits tumor-specific immunity [6]. At present, however, the processing and presentation pathway yielding the transfer of exogenous Hsp90-associated peptide antigens to MHC class I molecules is unknown.

We have investigated the role of exogenous Hsp90 to elucidate the mechanism of immunity induction via cross-presentation. We discuss here the mechanism of the anti-cancer immune response induced by exogenous Hsp90-antigen complexes.

### *Innate immune responses via HSP-APC interaction*

Recent evidence suggests that HSP are be involved in innate immune responses, i.e. activation of antigen-presenting cells (APC) such as DC and macrophages. Hsp70 [7] and Hsp60 [8] have been shown to be TLR4 ligands. DC induced from mouse myeloid cells are activated by gp96, and this leads to the up-regulation of CD86 and CD83 expression [9]. Thus, HSP are key players in the activation and induction of maturation of immature DC. These facts reinforce the idea that extracellular HSP act as a "danger signal", which alerts the innate immune system to sites of cell stress or necrosis. Local exposure to HSP from injured or necrotic cells would induce activation of DC and initiate the innate immune responses. At the same time, because HSP chaperone antigens derived from tumor or virus-infected cells, interaction of HSP and their receptors results in the uptake of HSP-antigen complexes, followed by processing of chaperoned antigens and their representation by MHC class I molecules on DC. When cells are exposed to severe stress, the amount of released HSP-antigen complexes is high, transmitting strong danger signals to DC and causing robust immune responses. Therefore, HSP as a danger signal is a rational system for immunosurveillance. In other words, HSP act as a conductor for orchestrating innate immunity and adaptive immunity (Figure 1).

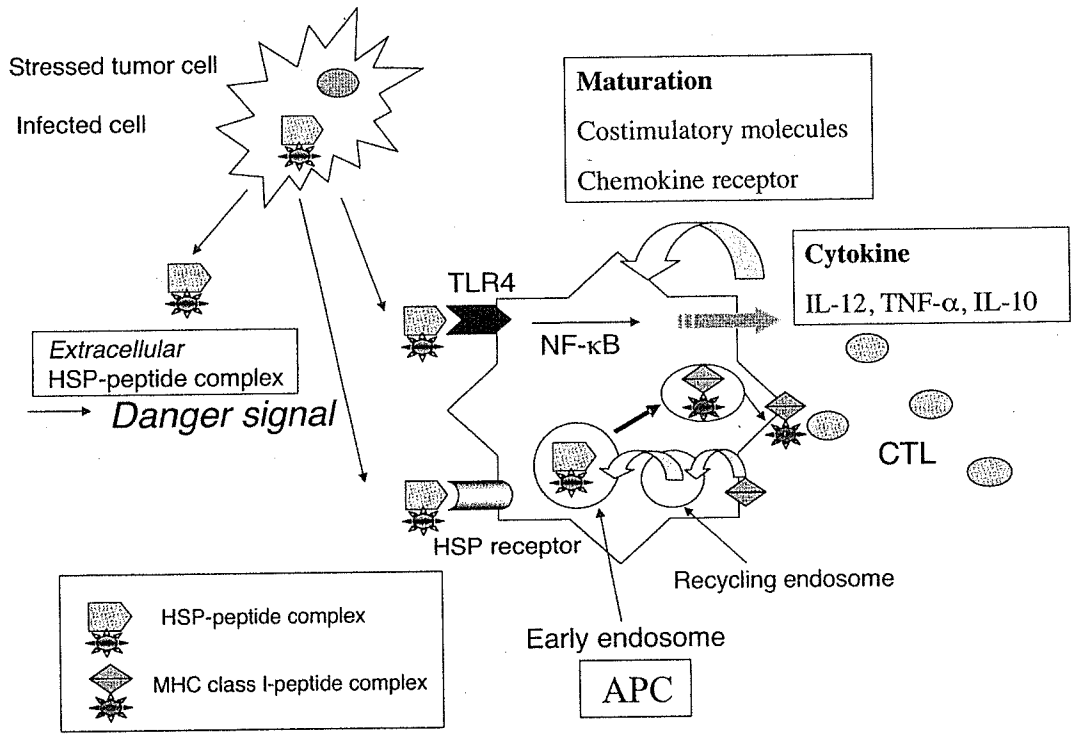


**Figure 1.** Role of extracellular HSP-peptide complex as a danger signal. HSP-peptide complexes are acquired by bone marrow-derived antigen presenting cells (APC) and are cross-presented to cytotoxic T lymphocytes (CTL). HSP-peptide complexes bind to Toll-like receptor (TLR) 4 and induce maturation and activation of dendritic cells (DC). On the other hand, HSP-peptide complexes also bind to HSP receptor, such as CD91, LOX-1, SR-A, on the DC, followed by endocytosis. Internalized HSP-peptide complexes are shuttled into MHC class I pathway via endosomal pathway, and induce CTL response. HSP-peptide complexes elicit both innate and adaptive immunity simultaneously, indicating that HSP-peptide complexes act as danger signal.

In harmony with this idea, HSP-peptide complex meditated cross-presentation can help to elucidate the missing link between endogenous antigen presentation and exogenous antigen presentation via the MHC class I pathway, emerging topics because it is believed that cross-presentation plays a very important role in basic tumor immunology and cancer immunotherapy.

***Overview: Exogenous HSP-antigen complex-mediated cross-presentation by DC***

Recent evidence has indicated that exogenous Hsp70- and gp96-chaperoned peptides can be cross-presented to CTL by DC in the context of MHC class I molecules [10-16]. The mechanism for HSP-mediated cross-presentation has been shown to be Hsp70- or gp96-peptide complex internalization via receptor-mediated endocytosis. CD91 [9,17,18], LOX-1 [19,20], CD40 [21] and SR-A [22] have been proved to be common receptors for HSP. Recently, Nicchitta *et al.* have shown that gp96 internalized by receptor-mediated endocytosis trafficks to an Fc receptor and MHC class I-positive endocytic compartment and does not access the ER of DC [23,24]. We have also shown that exogenously loaded tumor-derived Hsp70 is imported into the early endosome by endocytosis and does not reach the ER or lysosome [16]. The tumor-derived Hsp70-peptide complex is efficiently cross-presented to peptide-specific CTL by DC and this presentation is dependent on transporter-associated antigen processing (TAP) molecules. This fact suggests that processing and loading a peptide onto MHC class I requires translocation of the peptide from the endocytic compartment to the cytosolic pathway. Rodriguez et al. demonstrated that DC have a unique membrane transport pathway linking the lumen of endocytic compartments and the cytosol [25]. Thus, in DC, the exogenous HSP-chaperoned peptide in the endocytic compartment is released into the cytosol, where it follows the classical proteasome- and TAP-dependent class I pathway for presentation. Further studies to define the precise mechanisms for Hsp70- and gp96-chaperoned peptide trafficking may reveal a new paradigm for cross-presentation (Figure 2)

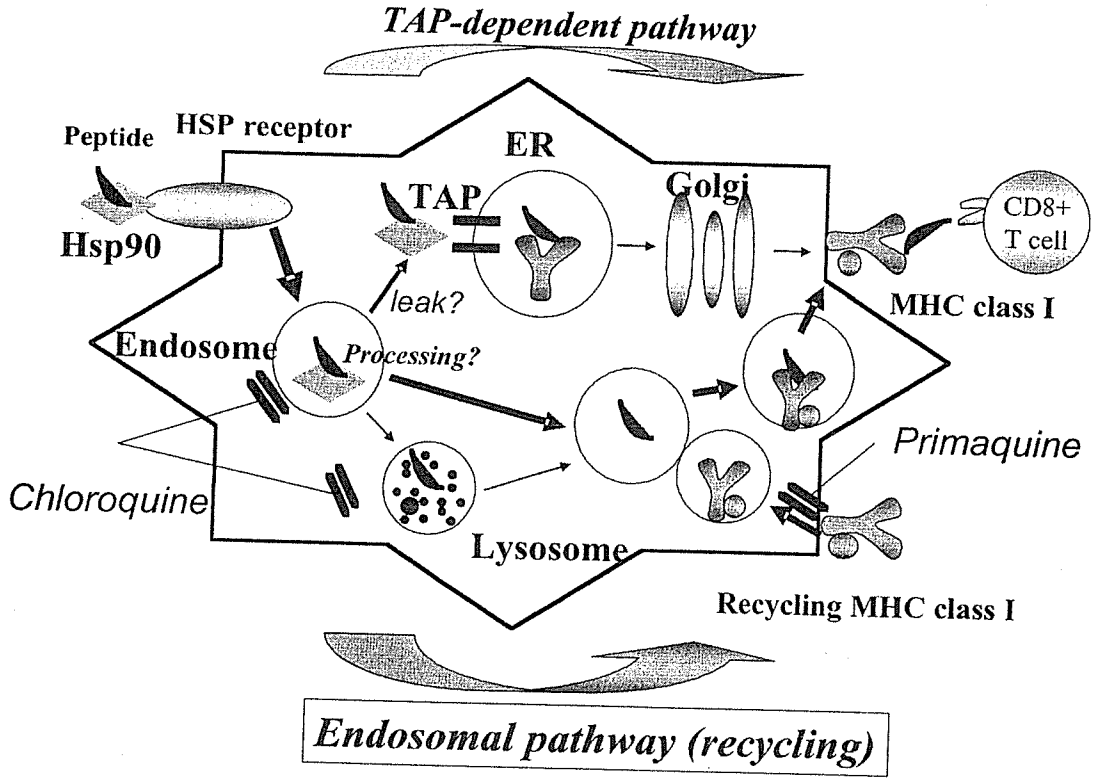

**Figure 2.** Possible pathway of Hsp90-antigen complex-mediated cross-presentation. Internalized Hsp90-antigen complexes through receptor-mediated endocytosis follow 2 distinct MHC class I pathways. (1) Internalized Hsp90-antigen complexes are transferred to the cytosol and imported into the ER in a TAP-dependent fashion. Hsp90-chaperoned antigens are degraded in the cytosol by proteasome and further trimmed by cytosolic peptidases. The resulting peptides are transported into the lumen of the ER for loading on newly synthesized MHC class I molecules. (2) Alternatively, internalized antigens chaperoned by Hsp90 are processed and loading in the endocytic pathway onto MHC class I molecules that are recycled from the plasma membrane, independently of TAP. To elucidate how Hsp90-antigen complex is processed transported for cross-presentation, DC derived from TAP knockout mouse, chloroquine for blocking the acdification of endosomal compartment, and primaquine for blocking the recycling membrane transport.

## Cross-presentation by exogenous Hsp90-peptide complex

Hsp90 is a one of most abundant proteins within cells and is overexpressed in many cancer cells. Therefore, once cancer cells become necrotic, much Hsp90 would be released from cells and might be a danger signal, subsequently eliciting cell-specific immune responses. It has been demonstrated that the tumor-derived Hsp90-peptide complex elicits tumor specific immunity. At present, however, the processing pathway yielding the transfer of exogenous Hsp90-associated peptide antigens to MHC class I molecules is unknown.

We examined the roles of Hsp90 in MHC class I-restricted cross-presentation using bone marrow-derived dendritic cells (DC) as APC. First, we tested whether Hsp90-peptide complexes reconstituted *in vitro* were uptaken and associated peptides presented in the context of MHC class I molecules by DC. To monitor the MHC class I antigen-processing pathway, we used Hsp90 reconstituted *in vitro* with the C-terminal extended version of VSV8 (RGYVYQGL), VSV-C (RGYVYQGLKSGNVSC: 15mer). The Hsp90-VSV-C peptide complex was cocultured with DC for 2 hours, followed by incubation with a VSV8-specific CTL clone. The culture supernatant was assayed for the production of IFN-γ. VSV-C-loaded Hsp90 was processed and presented by H-2K$^b$ and recognized by the VSV8-specific CTL clone but not Hsp90 or VSV-C alone. In the presence of an anti-H-2K$^b$ mAb during the presentation assay, the presentation of VSV8 to the specific CTL clone was completely abolished. These data suggested that HSP90-bound VSV-C peptides were processed to VSV8 within the cells with subsequently gained access to the MHC class I pathway. Intriguingly, this presentation occurred within 15 min, indicating that very rapid and efficient processing might be achieved within DC.

Next, we investigated whether the HSP90-mediated MHC class I pathway requires functional TAP molecules. To test this, we used DC derived from the TAP1-/- mouse. Surprisingly, DC from the TAP1-/- mouse could also process and present Hsp90-bound VSV-C peptides as efficiently as DC from the wild-type mouse.

We also tested another well-characterized H-2K$^b$-restricted OVA$_{257-264}$ antigen system, Hsp90 reconstituted *in vitro* with the C-terminal extended version of SL8 (OVA$_{257-264}$), SL8-C peptide (OVA$_{257-269}$: 13mer). The Hsp90-SL8-C peptide complex was cocultured with DC for 2 hours, followed by incubation with SL8-specific B3Z T cell hybridoma. As shown in Figure 3B, the Hsp90-SL8C peptide complex was processed and presented by H-2K$^b$ and recognized by B3Z T cell hybridoma, but not Hsp90 or SL8-C alone, in a TAP-independent manner. These experiments demonstrate that the TAP-independent pathway is used for Hsp90-mediated MHC class I presentation.

## HSP receptors expressed on antigen presenting cells

The existence of HSP receptors on APC has long been postulated because a microgram order of HSP elicits strong CD8+ T cell responses without any other adjuvant. Recently, CD91 (an α2-macroglobulin receptor), CD40 and scavenger receptor-A (SR-A) were proposed to serve as unique receptors responsible for the receptor-mediated endocytosis of HSP, but the molecular bases of such roles are still unclear. A definitive Hsp90 receptor has not yet been reported. In our system, competition assay revealed that some receptors expressed on DC were involved in Hsp90-mediated cross-presentation. We are currently investigating the Hsp90 receptor expressed on DC.

## Intracellular localization of endocytosed exogenous Hsp90-peptide complexes

Using laser confocal microscopy, we found that Hsp90-peptide complexes accumulate only in the endosome and do not reach the stage of the lysosome. We also examined whether Hsp90 accumulation in the endosome was due to temperature-dependent endocytosis. As expected, at 4°C, labeled Hsp90 remained on the cell surface, but internalization was evident after incubation at 37°C following a 10-min internalization period.

## The pathway for Hsp90-mediated cross-presentation

To investigate where the Hsp90-associated antigenic peptides bind to MHC class I molecules, we stained H-2K$^b$ molecules and exogenous Hsp90-peptide complexes. After 20 min of endocytosis, Alexa-labeled Hsp90 colocalized with endocytosed H-2K$^b$ molecules. The results showed the internalization and colocalization of H-2K$^b$ and internalized Hsp90 was evident in the early endosome. This finding suggested that HSP-bound peptides might be transferred to MHC class I molecules in the endosome, where recycled MHC class I molecules from the plasma membrane are available. The peptide-MHC class I complexes generated in the endosome are then transported to the cell surfaces of the DC, where specific CTL recognize them.

Recycling of endocytosed MHC class I molecules back to the cell surface has been observed. Some of the recycling MHC class I molecules can be loaded into endosomes with peptides derived from endocytosed molecules [26]. Therefore, to confirm whether this presentation really utilizes recycled MHC class I molecules, we treated DC with primaquine, which blocks the membrane recycling pathway. DC incubated in the presence of this drug could not present Hsp90-chaperoned SL8C-derived SL8 peptide. This result indicated that precursor peptides chaperoned by Hsp90 or processed peptides entered into recycling endosomes and transferred onto recycling MHC class I molecules, which returned to the cell surface and stimulated B3Z T cell hybridoma. To analyze the involvement of vacuolar acidification of endosomal compartments, DC were incubated with Hsp90-SL8C in the presence of chlorquine, a known inhibitor of acidification of endosomal compartments. Chloroquine strongly inhibited Hsp90-mediated presentation without affecting SL8 peptide presentation, showing that acidification of endosomal compartments is necessary for processing of Hsp90-chaperoned precursor peptides.

## Hsp90-chaperoned precursor peptides are processed by the endosomal protease

We used protease inhibitors to investigate the proteolytic processes involved in the Hsp90-mediated TAP-independent cross-presentation pathways. In wild-type DC, a broadly active cysteine protease inhibitor, leupeptin, almost completely inhibited the cross-presentation of Hsp90-SL8C. In contrast, the aspartic protease inhibitor pepstatin did not affect the cross-presentation. Cathepsins S, D and L are known to be the major cysteine proteases in endocytic compartments. We therefore examined the roles of various cathepsins in this pathway. Cathepsin D- and cathepsin L-specific inhibitors did not affect the cross-presentation, whereas a cathepsin S inhibitor completely blocked cross-presentation. Cathepsin S is a cysteine protease that is preferentially expressed in APC, including DC, macrophages, and B cells within endocytic compartments. Therefore, our data indicate that cathepsin S is a critical enzyme in TAP-independent Hsp90-mediated cross-presentation on MHC class I molecules and that the presented peptides are indeed generated in endosomal compartments.

## Advantages of Hsp90-antigenic protein complexes

As described above, we have shown that Hsp90-chaperoned precursor peptides are efficiently processed and presented by MHC class I molecules. To extend the range of HSP-based immunotherapy, we examined whether whole protein antigens chaperoned by Hsp90 were processed and presented by MHC class I molecules as well as class II molecules. The advantages of using protein antigens for cancer immunotherapy are that they can (1) provide an inherent polyvalent vaccine for CD8+ T cells, and (2) they include CD4+ helper epitopes, required for efficient CTL induction and proliferation. However, protein antigens themselves are not primarily immunogenic and therefore an immunostimulatory adjuvant is necessary for effective T cell responses. Given the well-known ability of HSP to forms complexes with naturally synthesized proteins, it is possible that Hsp90-protein antigen complexes could elicit antigen-specific CTL responses and Th responses as well. Therefore, we investigated the impact of Hsp90 on the presentation of exogenous protein antigens using ovalbumin (OVA) as a model antigen. We observed that the Hsp90-OVA complex generated in vitro was very efficiently and selectively presented via the MHC class I pathway both in vitro and in vivo, and that cross-presentation was involved in both TAP-dependent and -independent pathways. These results provide a rationale for the development of novel vaccination strategies for cancer immunotherapy.

### Hsp90-OVA complex is efficiently cross-presented by DC.

We next evaluated cross-presentation of the Hsp90-OVA protein complex. DC were pulsed with Hsp90 alone, free OVA, a simple mixture of the two or the two in a complex generated in vitro for 2 hrs at 37°C, then fixed, washed and cultured with B3Z CD8+ T cell hybridoma. The Hsp90-OVA complex elicited strong CTL responses, whereas Hsp90 or OVA alone did not lead to CTL responses. Notably, when we pulsed the simple mixture of

Hsp90 and OVA, we did not detect significant CTL responses. These results show that binding to Hsp90 is essential for cross-presentation of OVA.

## Hsp90-OVA complex is efficiently and selectively presented through MHC class I pathway

We also tested whether the Hsp90-OVA complex was presented through the MHC class II pathway, and elicited CD4+ T cell responses. DC from B6C3F1 were pulsed with free OVA or Hsp90-OVA complex for 2 hrs at 37°C, then fixed, washed and co-cultured with B3Z CD8+ T cell hybridoma or KZO CD4+ T cell hybridoma. Stimulation with free OVA led not to CTL responses but strong CD4+ T cell responses. In contrast, stimulation with the Hsp90-OVA complex elicited significantly weaker CD4+ T cell responses than free OVA, whereas it induced robust CTL responses. These findings suggest that the Hsp90-OVA complex is presented much more selectively through the MHC class I pathway than the MHC class II pathway.

To examine the differences in presentation efficacy between the Hsp90-OVA complex and free OVA, a pulse-chase experiment was performed. DC were pulsed with OVA alone or the Hsp90-OVA complex at 37°C, and harvested at different times from 10 min to 2 hrs, then fixed, washed and cultured with B3Z or KZO. B3Z responses were seen after 10 to 30 min of stimulation with the Hsp90-OVA complex (Figure), although no KZO responses were detected up to 1 hr with free OVA. These data demonstrate that cross-presentation of the Hsp90-OVA complex is more rapid and efficient than presentation of free OVA.

## Hsp90-antigen complex is cross-presented by DC *in vivo*

It is very important to show the efficacy of Hsp90-antigen complexes to stimulate naïve CD8+ T cells *in vivo*. We immunized mice with the Hsp90-OVA complex injected into a footpad. Then popliteal lymph-node derived CD11c-positive DC were harvested and cocultured with OVA-specific T cell hybridoma B3Z. DC from mice immunized with the Hsp90-OVA complex stimulated B3Z responses, suggesting that DC cross-presented the OVA-derived SL8 peptide in vivo. Furthermore, we immunized HLA A*2402 transgenic mice with a cancer-associated antigen survivin-2B-derived peptide [27,28]. After immunization with the Hsp90-survivin-2B peptide complex, efficient CTL induction was evident, whereas it was not after immunization with the peptide emulsified in IFA.

## Application of Hsp90-peptide complex for cancer immunotherapy

The development of an effective and safe cancer vaccine remains a high priority. Basically, strategies for cancer vaccines are divided in two categories. The first type is based on the synthesis of antigens by APC or their active delivery into the cytoplasm of these cells and exploits the "classical" cytosolic MHC class I pathway. The second type takes advantage of the capacity of cross-presentation of APC and is based on free or cell-associated antigens. We are now exploring the use of Hsp90-antigen complexes as cancer vaccines. Taking advantage of the extremely efficient cross-presentation by Hsp90-antigen complexes, we have examined the in vivo induction of CTL responses after treatment with Hsp90-antigen complexes. We previously reported that survivin and its splicing variant survivin-2B were

expressed abundantly in various tumor tissues and were suitable as target antigens for immunotherapy [27]. Subsequently, we identified an HLA-A24-restricted antigenic peptide, survivin-$2B_{80-88}$ (AYACNTSTL) recognized by CD8+ cytotoxic T lymphocytes (CTL) [28]. On the basis of these observations, we have started a phase I clinical study of survivin-2B peptide vaccination for patients with advanced colorectal cancer. To examine whether the Hsp90-mediated cross-presentation occurs in vivo, we immunized HLA-A*2402/$K^b$ transgenic mice with Hsp90-survivi-2B peptide complex. Spleen cells of mice immunized with Hsp90-survivin-2B complex showed significant cytotoxicity against survivin-2B-coated RMA-S-A*2402 cells, but not survivin-2B-coated RMA-S. In addition, we have established human survivin-2B transfected fibrosarcoma derived from HLA-A*2402/$K^b$ transgenic mouse. As expected, treatment with the Hsp90-survivin-2B peptide complex had a powerful therapeutic effect against established tumors as a result of its capacity to stimulate cytotoxic CD8+ T cell responses without any other adjuvant. The capacity of Hsp90-antigen complexes to elicit strong CTL responses is related to their ability to target APC as well as to escort chaperoned antigens into the alternative MHC class I pathway (cross-presentation).

## The fate of Hsp90-antigen complexes and HSP receptors

Herein we report that both peptide antigens and protein antigens are efficiently delivered to MHC class I processing and presentation pathways (cross-presentation) if they are complexed with Hsp90. Unexpectedly and interestingly, Hsp90-chaperoned protein antigens, which include MHC class II epitopes, were not presented by class II molecules. Although presentation of both types of antigen requires receptor-dependent endocytosis, the fates of the antigens are totally different. In the case of Hsp90-chaperoned proteins, both proteasomal processing and endosomal enzymes are involved in cross-presentation. Hence, identification of the Hsp90 receptor for cross-presentation is an essential issue. In addition, the fate of the internalized Hsp90 and its receptor should be examined. Our data demonstrate that the fate of Hsp90 chaperoned antigens is as follows: some are degraded in endosomal compartments (TAP-independent) while the rest escape to the cytosol where they are degraded by proteasomes to be presented for MHC class I cross-presentation (TAP-dependent).

## The mechanism for translocation of Hsp90-chaperoned antigens from endosomes to cytosol

The mechanism for escaping to the cytosol remains unknown, as is whether the Hsp90-antigen complex or its components separately escape to the cytosol. It is possible that the Hsp90-antigen complex first needs to be preprocessed in the endosomal compartments before being transferred to the cytosol to be further degraded by proteasomes. One possibility is that the mildly acidic pH in the endocytic compartments plays an important role for the transport of ingested antigens, and another is that delayed fusion with the late endosome/lysosome is important for the transport. Immature DC maintained mildly acidic pH in the endocytic compartment even after antigen uptake and could transport these antigens into the cytosol [29]. Chloroquine treatment inhibits the acidification of the endocytic compartments. Our data indicated that chloroquine treatment inhibited Hsp90-OVA presentation by DC in both TAP-dependent and -independent pathways. This suggested that antigen transport was dependent on mildly acidic pH-inducible machinery in the endocytic compartments of DC. However, a recent report showed that treatment with chlorquine or

$NH_4Cl$ enhanced the efficiency of cross-presentation [30]. These treatments accelerated export of exogenous soluble antigens from endocytic compartments to cytosol, thereby enhancing cross-presentation. The difference between ours results and theirs was the method of uptake by DC. In our case it occurred via receptor-mediated endocytosis, whereas they indicated that it occurred via phagocytosis or macropinocytosis. In addition, the regulators for the transport are still unclear. We are still on the road to the complete understanding of HSP-mediated immune regulation, and further studies will be required to elucidate the precise mechanism (Figure 3).

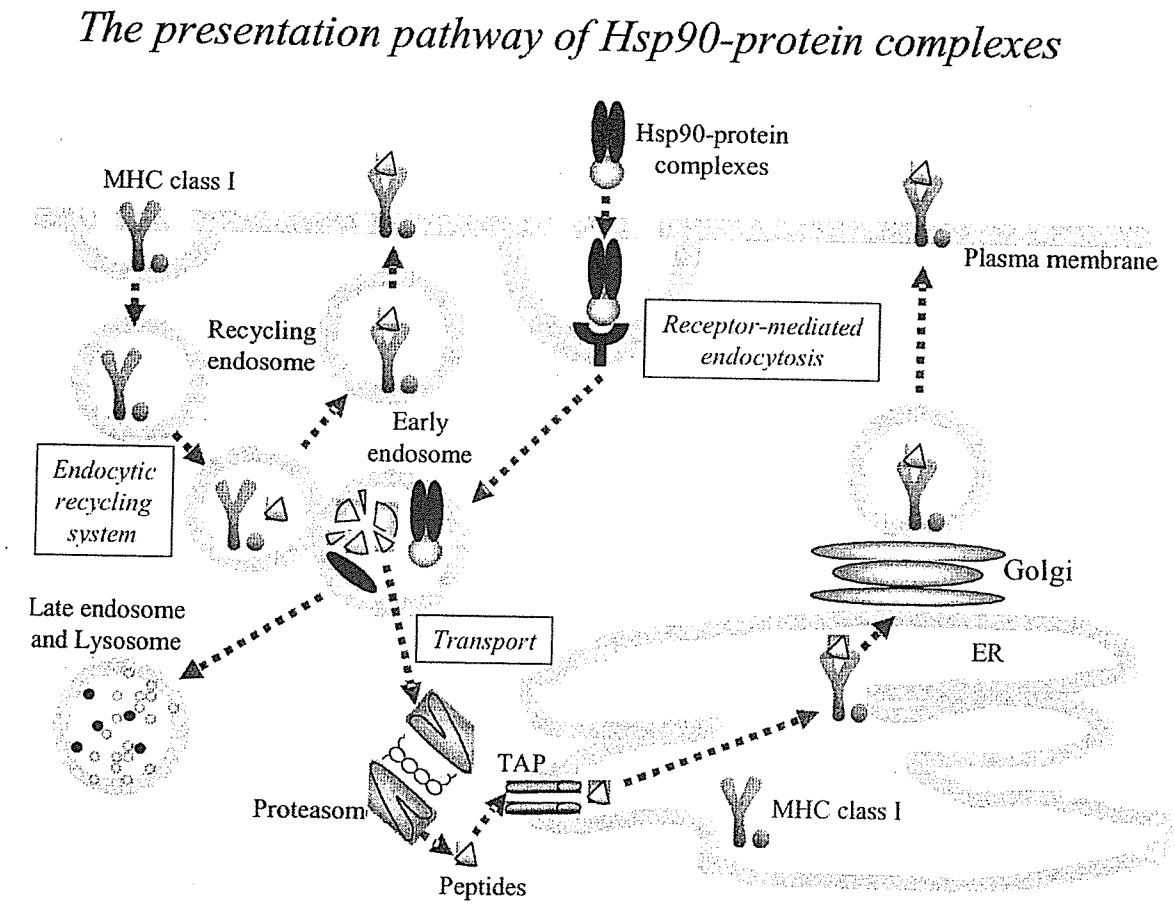


**Figure 3.** Route of access to the cytosol for internalized Hsp90-antigen complexes. Pathways for generation of Hsp90-chaperoned peptide-MHC class I complexes. Antigens are first cleaved endosomal protease, such as cathepsin S, and resulting peptide intermediates then enter into cytosol, followed by proteasomal degaradation. A small fraction of peptide intermediates are further trimmed by endosomal protease within endosomes, thereafter reach recycling endosomes and are loaded onto MHC class I molecules, which the return to plasma membrane occurs. It remains unclear what the mechanisms are by which they traverse the endosomal membrane or if it reflects the existence of a specific channel or a translocator.

## Why and how do HSP-antigen complexes skew the CD8+ T cell responses

## but not CD4+ T cell responses?

Ramirez et al. reported that a gp96-peptide complex elicited CD8+ T cell responses but not CD4+ T cell responses [31]. We have also observed that presentation of exogenous Hsp90-chaperoned peptides and protein antigens tends to drive the CD8+ T cell response. Although, protein antigens such as OVA protein contain both a CD8+ T cell epitope and CD4+ T cell epitope, if OVA is chaperoned by Hsp90, the Hsp90-OVA complex is taken up by receptor-mediated endocytosis and enters the cross-presentation pathway, followed by CD8+ T cell responses. In contrast, the soluble form of OVA protein is pinocytosed and follows the classical class II pathway. As for what drives Hsp90-antigen complexes into the cross-presentation pathway, the regulators for this transport are unclear and further studies will be required to elucidate the precise mechanism.

## Conclusion

Although HSP are primarily cytosolic proteins, they play an important role as a "danger signal" in the extracellular milieu on behalf of immune surveillance. Above all, Hsp90 is a one of the most abundant cytosolic proteins and elicits intriguingly efficient and rapid CTL responses. In this meaning, Hsp90 is a "smart and excellent guide" for the MHC class I cross-presentation pathway. A forthcoming issue is to illuminate the mechanism of the driving force toward the CD8+ T cell response mediated by HSP. This will clarify the impact of HSP as a danger signal for the etiologies of autoimmune diseases and tumor immunity.

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NPrCAP-magnetite with/without local heat generation can provide melanogenesis-targeted drug delivery system, kill primarily inoculated melanoma by non-apoptosis and reject secondarily inoculated melanoma by HSP-mediated immune reaction.

K Jimbow[1]*, T Takada[1], M Sato[1], A Sakemoto[1], Y Tamura[2], A Ito[3], H Honda[4], K Wakamatsu[5], S Ito[5] and T Yamashita[1]

*Departments of [1]Dermatology and [2]Pathology, Sapporo Medical University; [3]Department of Chemical Engineering, Kyusyu University; [4]Department of Biotechnology, Nagoya University; [5]Department of Chemistry, Fujita Health University, Toyoake, Japan.*

Management of malignant melanoma with distant metastases is a difficult challenge. Here we report our success in melanoma-targeted, drug delivery and chemo-thermo-immuno (CTI) therapy by utilizing a unique biological property, *i.e.*, melanogenesis. This is based upon our previous reports which showed that N-acetyl or N-propionyl derivative of cysteaminyl phenol (NAcCAP or NPrCAP) is a good substrate for tyrosinase, and selectively incorporated into melanoma cells and inhibit cell growth in the *in vivo* and *in vitro* systems. In addition, we have shown that magnetite encapsulated into cationic liposome (CML), when given intralesionally and exposed to alternating magnetic field (AMF), can inhibit melanoma growth by directly killing melanoma cells and stimulating immune reaction through heat shock protein (HSP). In our present *in vitro* and *in vivo* studies we have utilized NPrCAP as a selective drug delivery system (DDS) and anti-melanoma agent, and investigated the selectivity/specificity of NPrCAP for melanogenesis-targeted DDS and the anti-melanoma effect by combination of NPrCAP and magnetite particles (M), which were either encapsulated in non-cationic/ or cationic liposome complex (NPrCAP/ML or CML) or directly conjugated to magnetite (NPrCAP/M). The most efficient DDS with the fastest administration of NPrCAP with magnetite particles was obtained by NPrCAP/M which was a tyrosinase substrate, resulting in a selective uptake by melanoma cells compared to non-melanoma cells in both *in vitro* and *in vivo* systems. This selective uptake by melanoma cells was also observed by both local intralesional and systemic *ip* administration. Exposure of the *in vitro* cultured cells or *in vivo* animals with NPrCAP/M to AMF generated an immediate increase of the heat. NPrCAP/M with/without AMF was found to cause non-apoptotic cell death by DNA flow cytometry and agarose gel electrophoresis using a chimeric cDNA of p63/530 as an apoptosis control. Daily administration of NPrCAP/M for three times without AMF caused a statistically significant growth inhibition of melanoma cells (B16F1) grown *sc* in C57BL mice compared to control non-treatment groups. Repeated exposures to AMF in the same experimental group produced a steady generation of heat and complete abolishment of melanoma tissues inoculated *sc* into mice. Importantly when melanoma tissues inoculated *sc* primarily and received NPrCAP/M with or without AMF was removed and then another melanoma was inoculated at the opposite site of primarily inoculated lesion, the secondarily inoculated melanoma was rejected. This cytocidal effect of NPrCAP/M with and without AMF was found by both local intralesional and systemic *ip* administration, causing the HSP-mediated immune reaction against secondarily inoculated melanoma. Thus our NPrCAP/M provided a novel basis for developing melanoma-targeted DDS and CTI therapy.

# SULFUR-HOMOLOGUE OF MELANOGENESIS-SUBSTRATE WITH MAGNETITE (NPrCAP/M) CAN INHIBIT AND REJECT MELANOMA GROWTH

K Jimbow[1], T Yamashita[1], Y Tamura[2], N Sato[2], H Honda[3] and K Wakamatsu[4]
*[1]Department of Dermatology; [2]Department of Pathology I, Sapporo Medical University School of Medicine, Sapporo; [3]Bioprocess Engineering Laboratory, Department of Biotechnology School of Engineering, Nagoya University, Nagoya; [4]Department of Chemistry, Fujita Health University, Toyoake, Japan.*

In our previous reports we have shown that N-acetyl or N-propionyl derivative of cysteaminyl phenol (NAcCAP or NPrCAP) is a good substrate for tyrosinase, and selectively incorporated into melanoma tissues and inhibit melanoma cell growth. In this study, NPrCAP is conjugated with magnetite (M) either encapsulated into cationic liposome (CML) or neutral liposome (ML) or directly coupled. Magnetite can generate heat and kill cancer cells when exposed to alternating magnetic field (AMF). We investigated if NPrCAP serves as a selective drug delivery system (DDS) and if NPrCAP conjugated with magnetite can act as an anti-melanoma agent with chemo-thermo-immuno (CTI) therapeutitc effect when exposed to AMF. Selective melanoma-cytocidal effect was produced by NPrCAP which was directly conjugated to magnetite (NPrCAP/M), which also resulted in a selective uptake by melanoma cells compared to non-melanoma cells. Exposure to AMF generated a steady and immediate increase of the heat up to 46℃ in the *in vivo* system, which was found to cause non-apoptotic death of melanoma cells in the *in vitro* system. Administration of NPrCAP/M for three times without AMF caused a statistically significant inhibition of growth of melanoma cells (B16F1) grown *s.c.* in C57BL mice. Repeated exposures to AMF in the same experimental group produced a steady generation of heat and complete abolishment of melanoma tissues inoculated *s.c.* into mice. Further experiments with the excision of melanoma tissues at day 15 of post-inoculation and the above treatment showed the marked inhibition or complete rejection of melanoma cells which were inoculated at the opposite site of the initial melanoma inoculation. Thus our NPrCAP/M study showed our success in developing a novel, melanogenesis-targeted DDS and chemo-thermo-immuno (CTI) therapy for melanoma by AMF-generated heat.

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