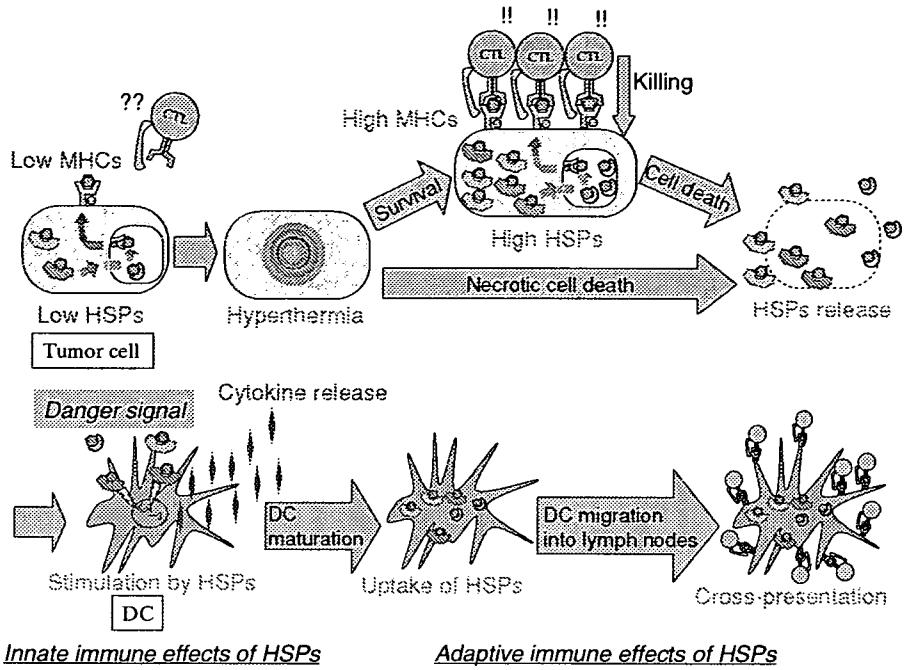

Fig. 3 Proposed scenario for the mechanism of anticancer immune induction by hyperthermia. Tumor cell with a low concentration of intracellular heat shock protein (HSP)-peptide complexes, decreased function of the endogenous antigen processing machinery, and a very low level of MHC class I-peptide complexes at the cell surface. Cytotoxic T lymphocytes (CTLs) are unable to locate tumor cells because of the low level of MHC class I expression, which causes poor immunogenicity of tumor cells. Hyperthermia treatment results in increased levels of intracellular HSP-peptide complexes, enhanced processing of endogenous antigens, and an increase in the density of MHC class I-peptide complexes at the cell surface. These tumor cells are then recognized directly by CTLs. Dying tumor cells, which are killed by the CTLs or by lethal hyperthermia treatment, release their intracellular contents, including HSP-peptide complexes. The released HSPs, acting as a "Danger signal," activate neighboring monocytes to produce proinflammatory cytokines and recruit antigen-presenting cells (APCs), including dendritic cells (DCs). The HSP-peptide complexes are taken up by DCs. Then DCs migrate into lymph nodes and are in turn presented to T cells via MHC class I and/or II antigens (cross-presentation).

served in an *in vitro* cytotoxicity assay using splenocytes in the cured mice treated with combination therapy, and the cured mice rejected a second challenge of B16 melanoma cells.

Since HSPs function as natural and powerful immunostimulants, recombinant HSP70 therapy[64] or HSP70 gene therapy[65] could be a possible approach. For recombinant HSP70 therapy, MCLs and recombinant mouse HSP70 were injected into melanoma nodules in C57BL/6 mice, which were subjected to hyperthermia at 43°C for 30 min. The combined treatment strongly inhibited tumor growth over a 30-day period and complete regression of tumors was observed in 20% (2/10) of mice. It was also found that systemic antitumor immunity was induced in the cured mice. For HSP gene therapy, a human HSP70 gene mediated by cationic liposomes was injected into a B16 melanoma nodule in C57BL/6 mice *in situ*. At 24 h after the injection of the HSP70 gene, MCLs were injected into melanoma nodules in mice, which were subjected to hyperthermia at 43°C for 30 min. Tumor growth was strongly arrested over a 30-day period after the combined treatment. Complete regression of tumors was observed in 30% (3/10) of mice, and systemic antitumor immunity was induced in the cured mice. These results suggest that this novel therapeutic strategy, combining the use of HSPs with hyperthermia using MCLs, may be applicable to patients with advanced malignancies.

Conclusions

We have reviewed recent progress in immunology and immunotherapy based on MCL-induced hyperthermia via HSP expression. From the point of view of immunology, evidence has been accumulating that HSPs play an important role in the immune response after hyperthermia treatment. Further investigations to elucidate the mechanisms are ongoing. Our preliminary results indicate that the depletion of HSPs in heated-cell lysate decreased the CTL response. In addition, the decreased level of CTL response associated with HSP70 depletion was higher than that in HSP90 (unpublished results), suggesting that HSP70 plays a pivotal role in the immune response induced by hyperthermia. For immunotherapy, we demonstrated that some modalities enhanced the

immune response induced by hyperthermia. We are conducting further studies in order to establish a novel cancer immunotherapy based on the concept of heat-controlled necrosis with HSP expression.

Acknowledgments

This work was partially supported by a Health and Labour Sciences Research Grants-in-Aid for Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare of Japan.

References

1) Dewey, W. C., Hopwood, L. E., Sapareto, L. A., and Gerweck, L. E. (1977). Cellular responses to combinations of hyperthermia and radiation. Radiology. 123: 463-474.
2) van der Zee, J. (2002). Heating the patient: a promising approach? Ann. Oncol. 13: 1173-1184.
3) Gordon, R. T., Hines, J. R., and Gordon, D. (1979). Intracellular hyperthermia. A biophysical approach to cancer treatment via intracellular temperature and biophysical alterations. Med. Hypotheses. 5: 83-102.
4) Jordan, A., Wust, P., Fähling, H., John, W., Hinz, A., and Felix, R. (1993). Inductive heating of ferrimagnetic particles and magnetic fluids: physical evaluation of their potential for hyperthermia. Int. J. Hyperthermia. 9: 51-68.
5) Wada, S., Tazawa, K., Furuta, I., and Nagae, H. (2003). Antitumor effect of new local hyperthermia using dextran magnetite complex in hamster tongue carcinoma. Oral Dis. 9: 218-223.
6) Mitsumori, M., Hiraoka, M., Shibata, T., Okuno, Y., Nagata, Y., Nishimura, Y., Abe, M., Hasegawa, M., Nagae, H., and Ebisawa, Y. (1996). Targeted hyperthermia using dextran magnetite complex: a new treatment modality for liver tumors. Hepatogastroenterology. 43: 1431-1437.
7) Jordan, A., Wust, P., Scholz, R., Tesche, B., Fahling, H., Mitrovics, T., Vogl, T., Cervos-Navarro, J., and Felix, R. (1996). Cellular uptake of magnetic fluid particles and their effects on human adenocarcinoma cells exposed to AC magnetic fields *in vitro*. Int. J. Hyperthermia. 12: 705-722.
8) Shinkai, M., Yanase, M., Suzuki, M., Honda, H., Wakabayashi, T., Yoshida, J., and Kobayashi, T. (1999). Intracellular hyperthermia for cancer using magnetite cationic liposomes. J. Magn. Magn. Mater. 194: 176-184.
9) Le, B., Shinkai, M., Kitade, T., Honda, H., Yoshida, J., Wakabayashi, T., and Kobayashi, T. (2001). Preparation of tumor-specific magnetoliposomes and their application for hyperthermia. J. Chem. Eng. Jpn. 34: 66-72.
10) Shinkai, M., Le, B., Honda, H., Yoshikawa, K., Shimizu, K., Saga, S., Wakabayashi, T., Yoshida, J. and Kobayashi, T. (2001). Targeting hyperthermia for renal cell carcinoma using human MN antigen-specific magnetoliposomes. Jpn. J. Cancer Res. 92: 1138-1145.
11) Ito, A., Kuga, Y., Honda, H., Kikkawa, H., Horiuchi, A., Watanabe, Y., and Kobayashi, T. (2004). Magnetite nanoparticle-loaded anti-HER2 immunoliposomes for combination of antibody therapy with hyperthermia. Cancer Lett. 212: 167-175.
12) Jordan, A., Scholz, R., Maier-Hauff, K., van Landeghem, F. K., Waldoefner, N., Teichgraeber, U., Pinkernelle, J., Bruhn, H., Neumann, F., Thiesen, B., von Deimling, A., and Felix, R. (2006). The effect of thermotherapy using magnetic nanoparticles on rat malignant glioma. J. Neurooncol. 78: 7-14.
13) Johannsen, M., Thiesen, B., Jordan, A., Taymoorian, K., Gneveckow, U., Waldofner, N., Scholz, R., Koch, M., Lein, M., Jung, K., and Loening, S. A. (2005). Magnetic fluid hyperthermia (MFH) reduces prostate cancer growth in the orthotopic Dunning R3327 rat model. Prostate. 64: 283-292.
14) Suzuki, M., Shinkai, M., Honda, H., and Kobayashi, T. (2003). Anticancer effect and immune induction by hyperthermia of malignant melanoma using magnetite cationic liposomes. Melanoma Res. 13: 129-135.
15) Ito, A., Tanaka, K., Honda, H., Abe, S., Yamaguchi, H., and Kobayashi, T. (2003). Complete regression of mouse mammary carcinoma with a size greater than 15 mm by frequent repeated hyperthermia using magnetite nanoparticles. J. Biosci. Bioeng. 96: 364-369.
16) Kawai, N., Ito, A., Nakahara, Y., Honda, H., Kobayashi, T., Futakuchi, M., Shirai, T., Tozawa, K., and Kohri, K. (2006). Complete regression of experimental prostate cancer in nude mice by repeated hyperthermia using magnetite cationic liposomes and a newly developed solenoid containing a ferrite core. Prostate. 66: 718-727.
17) Ito, A., Nakahara, Y., Fujioka, M., Kobayashi, T., Takeda, K., Nakashima, I., and Honda, H. (2005). Complete regression of hereditary melanoma in a mouse model by repeated hyperthermia using magnetite cationic liposomes. Jpn. J. Hyperthermic Oncol. 21: 179-189.
18) Yanase, M., Shinkai, M., Honda, H., Wakabayashi, T., Yoshida, J., and Kobayashi, T. (1998). Intracellular hyperthermia for cancer using magnetite cationic liposome: an *in vivo* study. Jpn. J. Cancer Res. 89: 463-469.
19) Matsuoka, F., Shinkai, M., Honda, H., Kubo, T., Sugita, T., and Kobayashi, T. (2004). Hyperthermia using magnetite cationic liposomes for hamster osteosarcoma. Biomagn. Res. Technol. 2: 3.
20) Matsuno, H., Tohnai, I., Mitsudo, K., Hayashi, Y., Ito, M., Shinkai, M., Kobayashi, T., Yoshida, J., and Ueda, M. (2001). Interstitial hyperthermia using magnetite cationic liposomes inhibit to tumor growth of VX-7 transplanted tumor in rabbit tongue. Jpn. J. Hyperthermic Oncol. 17: 141-149.
21) Yanase, M., Shinkai, M., Honda, H., Wakabayashi, T., Yoshida, J., and Kobayashi, T. (1998). Antitumor immunity induction by intracellular hyperthermia using magnetite cationic liposomes. Jpn. J. Cancer Res. 89: 775-782.
22) Lindquist, S. (1986). The heat-shock response. Ann. Rev. Biochem. 55: 1151-1191.
23) Ménoret, A. and Chandawarkar, R. (1998). Heat-shock protein-based anticancer immunotherapy: An idea whose time has come. Sem. Immunol. 25: 654-660.
24) Srivastava, P. K., Ménoret, A., Basu, S., Binder, R., and Quade, K. (1998). Heat shock proteins come of age: primitive functions acquired new roles in an adaptive world. Immunity. 8: 657-665.
25) Tamura, Y. and Sato, N. (2003). Heat shock proteins: chaperoning of innate and adaptive immunities. Jpn. J. Hyperthermic Oncol. 19: 131-139.
26) Srivastava, P. K. (2005). Immunotherapy for human cancer using heat shock protein-peptide complexes. Curr. Oncol. Rep. 7: 104-108.
27) Qiu, J., Li, G. W., Sui, Y. F., Song, H. P., Si, S. Y., and Ge, W. (2006). Heat-shocked tumor cell lysate-pulsed dendritic cells induce effective anti-tumor immune response *in vivo*. World J. Gastroenterol. 12: 473-478.
28) Kim, H. S., Choo, Y. S., Koo, T., Bang, S., Oh, T. Y., Wen, J., and Song, S. Y. (2006). Enhancement of antitumor immunity of dendritic cells pulsed with heat-treated tumor lysate in murine pancreatic cancer. Immunol. Lett. 103: 142-148.
29) Okamoto, M., Tazawa, K., Kawagoshi, T., Maeda, M., Honda, T., Sakamoto, T., and Tsukada, K. (2000). The combined effect against colon-26 cells of heat treatment and immunization with heat treated colon-26 tumour cell extract. Int. J. Hyperthermia. 16: 263-273.
30) Ito, A., Shinkai, M., Honda, H., Wakabayashi, T., Yoshida, J., and Kobayashi, T. (2001). Augmentation of MHC class I antigen presentation via heat shock protein expression by hyperthermia. Cancer Immunol. Immunother. 50: 515-522.
31) Ito, A., Shinkai, M., Honda, H., Yoshikawa, K., Saga, S., Wakabayashi, T., Yoshida, J., and Kobayashi, T. (2003). Heat shock protein 70 expression induces antitumor immunity during intracellular hyperthermia using magnetite nanoparticles. Cancer Immunol. Immunother. 52: 80-88.
32) Ito, A., Honda, H., and Kobayashi, T. (2006). Cancer immunotherapy based on intracellular hyperthermia using magnetite nanoparticles: a novel concept of "heat-controlled necrosis" with

heat shock protein expression. Cancer Immunol. Immunother. 55: 320-328.
33) Srivastava, P. K. and Maki, R. G. (1991). Stress-induced proteins in immune response to cancer. Curr. Top. Microbiol. Immunol. 167: 109-123.
34) Srivastava, P. K. and Heike, M. (1991). Tumor-specific immunogenicity of stress-induced proteins: convergence of two evolutionary pathways of antigen presentation? Semin. Immunol. 3: 57-64.
35) Srivastava, P. K., Udono, H., Blachere, N. E., and Li, Z. (1994). Heat shock proteins transfer peptides during antigen processing and CTL priming. Immunogenetics. 39: 93-98.
36) Wells, A. D., Rai, S. K., Salvato, M. S., Band, H., and Malkovsky, M. (1998). Hsp72-mediated augmentation of MHC class I surface expression and endogenous antigen presentation. Int. Immunol. 10: 609-617.
37) Wells, A. D. and Malkovsky, M. (2000). Heat shock proteins, tumor immunogenicity and antigen presentation: an integrated view. Immunol. Today. 21: 129-132.
38) Basu, S., Binder, R. J., Ramalingam, T., and Srivastava, P. K. (2001). CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity. 14: 303-313.
39) Becker, T., Hartl, F. U., Wieland, F. (2002). CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. J. Cell Biol. 158: 1277-1285.
40) Asea, A., Rehli, M., Kabingu, E., Boch, J. A., Bare, O., Auron, P. E., Stevenson, M. A., and Calderwood, S. K. (2002). Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J. Biol. Chem. 277: 15028-15034.
41) Binder, R. J., Vatner, R., and Srivastava, P. (2004). The heat-shock protein receptors: some answers and more questions. Tissue Antigens. 64: 442-451.
42) Asea, A., Kraeft, S. K., Kurt-Jones, E. A., Stevenson, M. A., Chen, L. B., Finberg, R. W., Koo, G. C., and Calderwood, S. K. (2000). HSP70 stimulates cytokine production through a CD14-dependent pathway, demonstrating its dual role as a chaperon and cytokine. Nat. Med. 6: 435-442.
43) Basu, S., Binder, R., Suto, R., Anderson, K. M., and Srivastava, P. K. (2000). Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NFkB pathway. Int. Immunol. 12: 1539-1546.
44) Asea, A., Kabingu, E., Stevenson, M. A., and Calderwood, S. K. (2000). HSP70 peptide-bearing and peptide-negative preparations act as chaperokines. Cell Stress Chaperones. 5: 425-431.
45) Lehner, T., Bergmeier, L. A., Wang, Y., Tao, L., Sing, M., Spallek, R., and van der Zee, R. (2000). Heat shock proteins generate beta-chemokines which function as innate adjuvants enhancing adaptive immunity. Eur. J. Immunol. 30: 594-603.
46) Panjwani, N. N., Popova, L., and Srivastava, P. K. (2002). Heat shock proteins gp96 and hsp70 activate the release of nitric oxide by APCs. J. Immunol. 168: 2997-3003.
47) Singh-Jasuja, H., Scherer, H. U., Hilf, N., Arnold-Schild, D., Rammensee, H. G., Toes, R. E., and Schild, H. (2000). The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. Eur. J. Immunol. 30: 2211-2215.
48) Noessner, E., Gastpar, R., Milani, V., Brandl, A., Hutzler, P. J., Kuppner, M. C., Roos, M., Kremmer, E., Asea, A., Calderwood, S. K., and Issels, R. D. (2002). Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells. J. Immunol. 169: 5424-5432.
49) Fuchs, E. J. and Matzinger, P. (1996). Is cancer dangerous to the immune system? Semin. Immunol. 8: 271-280.
50) Matzinger, P. (2002). The danger model: a renewed sense of self. Science. 296: 301-305.
51) Buttiglieri, S., Galetto, A., Forno, S., De Andrea, M., and Matera, L. (2003). Influence of drug-induced apoptotic death on processing and presentation of tumor antigens by dendritic cells. Int. J. Cancer. 106: 516-520.
52) Labarriere, N., Bretaudeau, L., Gervois, N., Bodinier, M., Bougras, G., Diez, E., Lang, F., Gregoire, M., and Jotereau, F. (2002). Apoptotic body-loaded dendritic cells efficiently cross-prime cytotoxic T lymphocytes specific for NA17-A antigen but not for Melan-A/MART-1 antigen. Int. J. Cancer. 101: 280-286.
53) Yonezawa, M., Ohtsuka, T., Matsui, N., Tsuji, H., Kato, K. H., Moriyama, A., and Kato, T. (1996). Hyperthermia induces apoptosis in malignant fibrous histiocytoma cells *in vitro*. Int. J. Cancer. 66: 347-351.
54) Todryk, S., Melcher, A. A., Hardwick, N., Linardakis, E., Bateman, A., Colombo, M. P., Stoppacciaro, A., and Vile, R. G. (1999). Heat shock protein 70 induced during tumor cell killing induces Th1 cytokines and targets immature dendritic cell precursors to enhance antigen uptake. J. Immunol. 163: 1398-1408.
55) Asea, A. (2007). Mechanism of HSP72 release. J. Biosci. 32: 579-584.
56) Lancaster, G. I. and Febbraio, M. A. (2005). Exosome-dependent trafficking of HSP70: a novel secretory pathway for cellular stress proteins. J. Biol. Chem. 280: 23349-23355.
57) Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Ricciardi-Castagnoli, P., Raposo, G., and Amigorena, S. (1998). Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat. Med. 4: 594-600.
58) Escola, J. M., Kleijmeer, M. J., Stoorvogel, W., Griffith, J. M., Yoshie, O., and Geuze, H. J. (1998). Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. J. Biol. Chem. 273: 20121-20127.
59) Fleshner, M., Campisi, J., Amiri, L., and Diamond, D. M. (2004). Cat exposure induces both intra- and extracellular Hsp72: the role of adrenal hormones. Psychoneuroendocrinology. 29: 1142-1152.
60) Ito, A., Tanaka, K., Kondo, K., Shinkai, M., Honda, H., Matsumoto, K., Saida, T., and Kobayashi, T. (2003). Tumor regression by combined immunotherapy and hyperthermia using magnetic nanoparticles in an experimental subcutaneous murine melanoma. Cancer Sci. 94: 308-313.
61) Ito, A., Fujioka, M., Tanaka, K., Kobayashi, T., and Honda, H. (2005). Screening of cytokines to enhance vaccine effects of heat shock protein 70-rich tumor cell lysate. J. Biosci. Bioeng. 100: 36-42.
62) Tanaka, K., Ito, A., Kobayashi, T., Kawamura, T., Shimada, S., Matsumoto, K., Saida, T., and Honda, H. (2005). Intratumoral injection of immature dendritic cells enhances antitumor effect of hyperthermia using magnetic nanoparticles. Int. J. Cancer. 116: 624-633.
63) Tanaka, K., Ito, A., Kobayashi, T., Kawamura, T., Shimada, S., Matsumoto, K., Saida, T., and Honda, H. (2005). Heat immunotherapy using magnetic nanoparticles and dendritic cells for T-lymphoma. J. Biosci. Bioeng. 100: 112-115.
64) Ito, A., Matsuoka, F., Honda, H., Kobayashi, T. (2004). Antitumor effects of combined therapy of recombinant heat shock protein 70 and hyperthermia using magnetic nanoparticles in an experimental subcutaneous murine melanoma. Cancer Immunol. Immunother. 53: 26-32.
65) Ito, A., Matsuoka, F., Honda, H., and Kobayashi, T. (2003). Heat shock protein 70 gene therapy combined with hyperthermia using magnetic nanoparticles. Cancer Gene Ther. 10: 918-925.

Ann. Cancer Res. Therap. Vol. 15, No. 2, pp. 41-49, 2007

Extracellular heat shock proteins (eHSPs) pilot exogenous antigen into cross-presentation pathway: A superguide from extracellular world to intracellular tour

Yasuaki Tamura, Goro Kutomi, Jun Oura, Toshihiko Torigoe, Noriyuki Sato

Department of Pathlogy, Sapporo Medical University, School of Medicine

Abstract

Heat shock proteins (HSPs) are intracellular chaperone, some of which function as immune adjuvants as well as danger signals for immune system when released into extracellular milieu. These HSPs, along with their client polypeptides, are specifically bound by receptors on antigen presenting cells (APCs). This leads to APC differentiation along with delivery of the chaperoned peptides for cross-presentation to T cells. HSP-APC interactions occur through several receptors that mediate endocytosis or signal transduction. Most importantly, HSP associated antigens are forced to enter the cross-presentation pathway by APCs, resulting in $CD8^+$ T cell activation. These unique features of HSPs for the generation of immune response will be discussed.

Keywords: Heat shock protein, Hsp90, Cross-presentation, Antigen presenting cell, MHC class I molecule

(Received August 13, 2007; Accepted August 31, 2007)

Introduction: HSP-mediated cross-presentation by antigen-presenting cells

It has been well demonstrated that immunization with tumor-derived HSPs or HSP complexed with an antigen peptide elicits tumor- or antigen-specific $CD8^+$ T cell responses. Above all, Hsp70- and gp96-antigen complexes are well-studied and have been shown to be immunogenic and potent in stimulating the generation of tumor-specific CTLs. Hsp70- and gp96-based vaccines have been tested in early-phase clinical trials in solid tumors as well as in lymphoma and leukemias; all showed minimal toxicity and potential efficacy[1-3]. Phase III clinical trials using tumor-derived Hsp70 and gp96 as vaccines are ongoing for melanoma and renal cell carcinoma.

The ability of HSPs to facilitate the cross-presentation of MHC class I-restricted epitopes and to prime $CD8^+$ T cell effector responses is well established[4,5]. Although, immunized HSPs are exogenous antigens, these HSP-antigen complexes can gain access to the class I antigen presentation pathway, resulting in cross-presentation. The immune response has been attributed to the ability of HSPs to form stable complexes with tumor-derived antigenic peptides, thereby facilitating the cross-presentation of MHC class I-restricted epitopes and priming of $CD8^+$ T cell responses.

Dendritic cells (DCs) are main conductor for efficient cross-presentation. Recent reports have shown that antigen-presenting cells (APCs) such as dendritic cells can internalize HSPs by receptor-mediated endocytosis and direct chaperoned proteins/peptides into the intracellular pathway for MHC class I-restricted presentation to $CD8^+$ T cells, concomitant with the induction of dendritic cell maturation and cytokine secretion. In fact, some HSP receptors expressed on APCs have recently been identified. CD91[6], LOX-1[7], CD40[8] and SR-A[9] have been proved to be common receptors for HSPs. However, the underlying mechanism for efficient cross-presentation, in particular, how the HSP-antigen complex can enter the MHC class I pathway, remains unclear.

Furthermore, recent studies have shown that HSP-peptide complexes can also lead to antigen presentation on MHC class II molecules, thus activating $CD4^+$ T cells[10,11]. Therefore, it is possible that uptake of HSP-peptides complexes leads to antigen presentation on both MHC class I and class II molecules on dendritic cells, thus activating $CD8^+$ CTL as well as $CD4^+$ T cells. However, Shild et al. have reported that, although antigen peptides chaperoned by gp96 can be presented in the context of both MHC class I and class II molecules, immunization with gp96 elicits CD8-biased T cell responses[12]. Doody et al have also demonstrated same results[5]. Therefore, it is essential to know the effects of the HSP-antigen complex on tumor antigen presentation via class

Correspondence to: Yasuaki Tamura, Department of Pathlogy, Sapporo Medical University, School of Medicine

I and class II pathways in vivo because such knowledge is crucial for the development of effective HSP-based immunotherapies, especially in the case of protein antigens in association with HSPs.

In contrast to Hsp70 and gp96, the role of Hsp90 in cross-presentation remains unclear. Hsp90 is the most abundant protein in the cytoplasm; therefore it is assumed that Hsp90 also plays an important role in cross-presentation. However, it is not clear whether Hsp90 is involved in tumor immunity. Udono reported, for the first time, that immunization with Hsp90 purified from tumor elicited tumor-specific CTL responses[13]. Very recently, Kunisawa and Shastri reported that Hsp90 chaperoned C-terminal flanking antigenic peptides[14]. These results have led to much interest in the importance of Hsp90 in antigen presentation. Taking these facts into consideration, it is conceivable that Hsp90 does participate in antigen presentation and possibly cross-presentation. In this paper, we will discuss the mechanism of HSP-mediated cross-presentation and the players involved in this intriguing immune response.

HSPs act as danger signals to the immune system through HSP receptors expressed on APCs.

It is suggested that extracellular HSPs (eHSPs) act as "danger signals" to the immune system in the case of life-threatening events (Fig. 1). When viral infection or tumor cell damage occurs, cell-associated antigens, such as HSPs-antigen complexes are released into the extracellular milieu, APCs such as dendritic cells and macrophages, detect the signals through certain receptors, resulting in intracellular signaling[15]. HSP receptors are divided into 2 categories, one is toll-like receptor (TLR) for mainly signaling for DC maturation and activation, and the other is endocytic receptors for cross-presentation. TLR-2 has been shown to be the receptor for Hsp70[16]. TLR-4 is the receptor for Hsp70[16,17]. However, doubts were raised as to what extent this effect was due to lipopolysaccharide contaminations of the HSP preparations. It is required re-examination for this phenomenon using HSPs, nominally endotoxin-free. In contrast, HSP-specific endocytic receptors expressed on the APCs, including CD91 for gp96, Hsp70 and Hsp90[6,18], SR-A for gp96 and calreticulin[9], LOX-1 for Hsp70[7], and CD40 for Hsp70[19], were identified. However, it is still unknown how HSP-antigen complexes are transported and where HSP releases chaperoned antigens. What is the fate of HSPs after endocytosis? What actors are responsible for translocating the antigen from the endosome to cytosol? Additional studies will be required to understand these unsolved issues.

The roads to MHC class I presentation

MHC class I molecules principally present peptides derived from endogenous protein to cytotoxic T cells. However, in certain antigen-presenting cells, peptides derived from exogenous antigens are also presented by MHC class I molecules. At least four independent pathways of protein processing and subsequent peptide presentation by class I molecules have been described. The dominant pathway uses endogenously synthesized proteins that have been processed in the cytosol by proteasomes. Peptides are transferred by transporter-associated antigen processing (TAP) to the ER where they bind in the grooves of nascent MHC class I molecules. The peptide/class I MHC heavy chain/β2m complex is then transported via the Golgi apparatus to the cell surface. In second and third pathways, exogenous antigens are internalized and processed into peptides that are transported to the ER to bind MHC class I. One is the cytosolic leak of internalized antigens by phagocytosis, macropinocytosis, and enocytosis, resulting in degradation by proteasomes[20,21]. Degraded peptides are then transported through TAP into the ER. However, the mechanism for the translocation of exogenous antigens to cytosol and players involved in this translocation remain unknown. Another pathway is via ER-phagosome fusion. When exogenous antigens are engulfed into phagosomes, the phagosomal membrane and ER membrane fuse with each other, forming ER-phagosome compartments[22,23]. These ER-phagosome fusion compartments involve TAP molecules and proteasomes outside of the membrane[24]. Phagocytosed antigens are pumped out the ER-phagosome fusion compartment through sec61. This is called the ER-associated degradation (ERAD) mechanism. Then proteasomes, which are attached to the outer face of the membrane of ER-phagosome fusion, degrade antigens into peptides, followed by entry into the ER again through TAP molecules, and the resulting antigen peptides bind to MHC class I molecules[25,26]. In contrast, at least some exogenous peptides or proteins appear to reach MHC class I through a pathway completely independent of the ER. In this fourth pathway, endosomal processing and endocytosed class I MHC molecules may be involved. MHC class I molecules have been shown to internalize from the cell surface in T cells, B cells, fibroblasts, and macrophages. Recycling of endocytosed class I MHC molecules back to the cell surface has also been observed[27]. The endocytosis and recycling of class I MHC may facilitate peptide exchange, allowing class I MHC molecules to bind multiple peptides in one lifetime. Antigen presentation mediated by the latter three types mentioned above is called cross-presentation, allows display of exogenous antigens in the context of MHC class I molecules. This is particularly important in

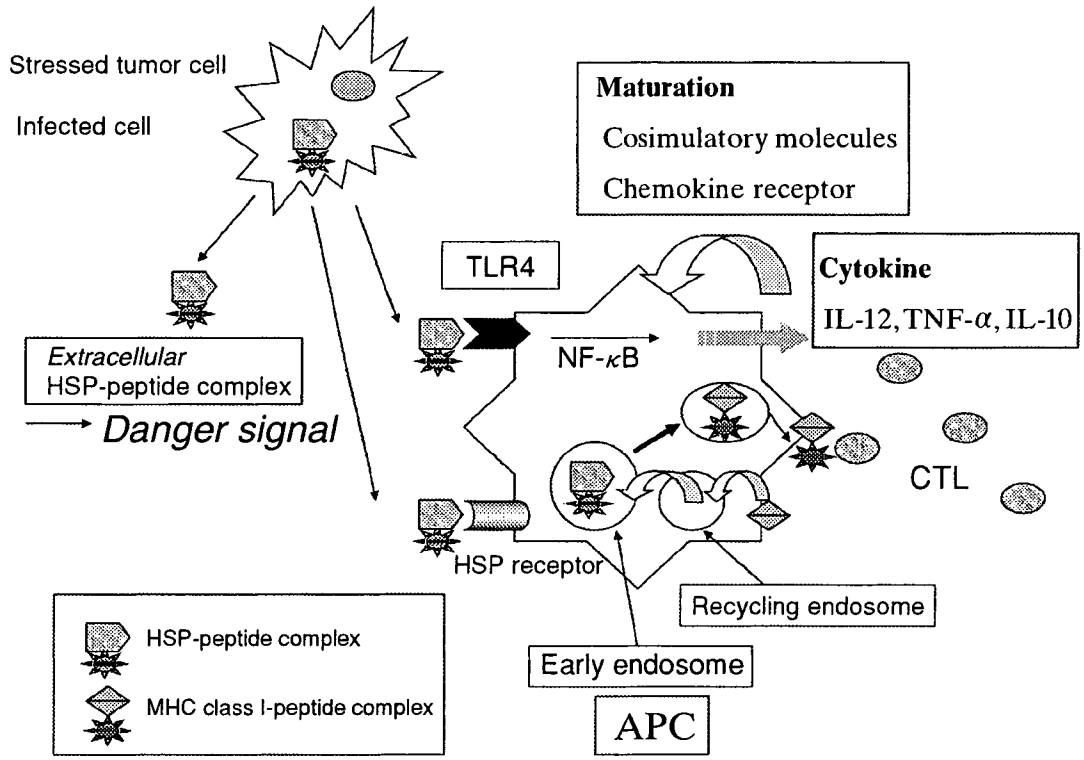

Fig. 1 **Role of extracellular HSP-peptide complex as a danger signal.** HSP-peptide complexes are acquired by bone marrow-derived antigen presenting cells (APC) and are cross-presented to cytotoxic T lymphocytes (CTL). HSP-peptide complexes bind to Toll-like receptor (TLR) 4 and induce maturation and 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.

host defense against infectious diseases and cancers that cannot access the classical pathway for MHC class I presentation. Moreover, for the development of the efficient cancer vaccine, it is necessary to establish the methods for delivering exogenous cancer vaccine into cross-presentation pathway.

Internalization of exogenous antigens may allow cell fragments, intracellular pathogens and proteins to be degraded in the endocytic pathway by mechanisms involving reduction, unfolding and lysosomal proteolysis. Such a process could contribute to cross-presentation by facilitating the exchange of previously loaded MHC class I-associated peptides for newly generated peptides derived from exogenous proteins. Cycloheximide treatment, which blocks the biosynthetic pathway of MHC I, indicated that the class I present in the endocytic compartments was derived from the cell surface. We have confirmed the presence of MHC class I in the endocytic compartments of murine bone marrow-derived DCs. Furthermore, as described recently, we have demonstrated that co-localization of the receptor-mediated endocytosed exogenous Hsp70/90-antigen complex with MHC class I in the early endosomes of the DCs. These observations suggest that antigens derived from the exogenous Hsp70/90 may be loaded onto recycling MHC class I, after which the MHC class I/peptide complex is transported to the cell surface.

Impact on HSP-antigen complex in a cross-presentation pathway

Considering the significance of the HSP-peptide complex in cross-presentation, this model suggests that a 'pre-processed antigen' would be required as it would be inefficient for a whole protein to be degraded non-specifically within the endocytic compartments by resident catabolic enzymes. Not only would this be a slow process, it would be by chance alone that an appropriate peptide capable of binding the MHC class I groove would be generated. The notion of such preprocessed antigens fits well with a role for HSPs as chaperones for peptide antigens. Proteins synthesized within the cell would be processed within the endogenous class I antigen presentation pathway leading to the generation of

HSP-peptide complexes. These complexes are ideal chaperones of antigenic peptides for the transfer of antigens to DCs for a number of reasons. Once generated within the cell, the HSP-peptide complex might be released into the extracellular milieu during cell necrosis because of viral infection and intervention of cancer, resulting in taking-up by the immature DC and acting as a danger signal. At the same time, antigenic peptides chaperoned by HSPs are efficiently presented in the context of MHC class I molecules and immediately activate the host's immune responses. As described earlier, Hsp90 binds precursor (pre-processed) peptides generated in the cells and thus, endosomal processing is a suitable mechanism for pre-processed peptides. HSP would also serve to protect the peptide antigen from degradation upon entry into the endocytic compartment of the DC. In addition, an as yet uncharacterized lysosomal enzyme may play a role in the processing of internalized antigens for generation of MHC I epitopes. Recently, Rock et al. have demonstrated that DC-restricted cathepsin S plays an important role in the processing of exogenous antigens for the generation of MHC I antigenic determinants in the early endosome[28].

Significance of cross-presentation in vivo: impact on epitope generation via endosomal processing and proteasomal processing

Although DCs are capable of using an endocytic exchange mechanism to create MHC class I-peptide complexes, typical somatic cells have only the classical pathway for the generation of MHC class I-presenting peptides. For $CD8^+$ T cells induced by cross-presentation to be functionally useful against pathogen-infected cells, it would seem that the epitope generation mechanism used should be the same as those in classical MHC class I processing. An endosomal exchange mechanism in which peptides are generated by different proteases in radically different conditions from those in the endogenous pathway, therefore seems unlikely to contribute substantially to the $CD8^+$ T cell repertoire. Cytoplasmic processing, including proteasomal proteolysis, and ER-based trimming would be expected to be involved to generate the same peptide sequences as those made by nonhematopoietic cells. In fact, although partial proteolysis may occur in the endocytic pathway, extensive experimental evidence suggests that exogenous antigens must reach the cytoplasm to be efficiently cross-presented. In DCs, the presentation of exogenous antigens is unaffected by both chloroquine and inhibitors of lysosomal proteolysis. Exogenous antigen presentation is, however, highly sensitive to specific inhibitors of the proteasome, indicating that cytoplasmic proteolysis is the main form of epitope generation in the cross-presentation pathway. In contrast, the HSP-mediated cross-presentation pathway has been shown to involve both a proteasomal pathway and an endocytic-recycling pathway. We have demonstrated that a tumor-derived Hsp70-peptide complex is efficiently cross-presented to peptide-specific CTLs by DCs and this presentation is dependent on TAP molecules. In addition, in vitro generated Hsp70- and gp96-antigen complexes have been shown to be cross-presented via a TAP-dependent parhway[29-31]. This fact suggests that processing and loading a peptide onto MHC class I requires translocation of the antigen from the endocytic compartment to the cytosolic pathway. Rodriguez et al. demonstrated that DCs have a unique membrane transport pathway linking the lumina of endocytic compartments and the cytosol[20]. Thus, in DCs, the exogenous HSP-chaperoned antigen 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.

Cross-presentation of exogenous Hsp90-peptide complex by dendritic cells

Hsp90 is a one of the 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 (DCs) as APCs[32]. First, we tested whether Hsp90-peptide complexes reconstituted *in vitro* were taken-up and associated peptides presented in the context of MHC class I molecules by DCs. 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) to moniter the processing of the precursor peptide. The Hsp90-VSV-C peptide complex was cocultured with DCs 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 $H\text{-}2K^b$ and recognized by the VSV8-specific CTL clone, but not Hsp90 or VSV-C alone. In the presence of an anti-$H\text{-}2K^b$ 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 (Fig.2A).

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

We also tested another well-characterized H-2K^b-restricted OVA$_{258\text{-}265}$ antigen system, Hsp90 reconstituted *in vitro* with the C-terminal extended version of SL8 (OVA$_{258\text{-}265}$), SL8-C peptide (OVA$_{258\text{-}270}$: 13mer). The Hsp90-SL8-C peptide complex was cocultured with DCs for 2 hours, followed by incubation with SL8-specific B3Z T cell hybridoma. As shown in Fig. 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 (Fig. 2B). These experiments demonstrate that a TAP-independent pathway is used for Hsp90-mediated MHC class I presentation.

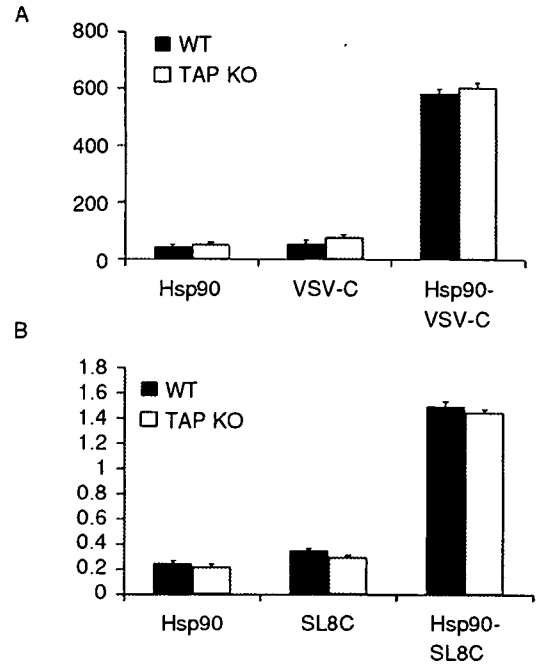

Fig. 2 **Precursor peptides chaperoned by Hsp90 are cross-presented via a TAP-independent pathway.** A. Hsp90-VSV-C complex, Hsp90 or VSV-C was loaded to BMDCs for 2 h and a VSV8-specific CTL clone was added. IFN-γ in the culture supernatant was measured by ELISA. BMDCs from the TAP$^{-/-}$ mouse could also process and present Hsp90-chaperoned VSV-C peptides efficiently as compared to BMDCs from the wild type mouse. B. SL8 precursor peptide, SL8C was also processed and cross-presented by BMDCs via a TAP-independent pathway.

Intracellular localization of endocytosed exogenous Hsp90-peptide complexes

Using laser confocal microscopy, we found that Hsp90-peptide complexes accumulated only in the endosome and did 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. According to a competition assay, DCs express Hsp90 receptor on the cell surfaces. Identification of receptor (s) will be necessary to elucidate the mechanism responsible for cross-presentation of exogenous Hsp90-antigen complexes.

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 co-localized with endocytosed H-2K^b molecules. The results showed the internalization and co-localization of H-2K^b and 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 DCs, where specific CTLs recognize them.

Recycling of endocytosed MHC class I molecules back to the cell surface has been observed. Some of the recycled MHC class I molecules can be loaded into endosomes with peptides derived from endocytosed molecules[27]. Therefore, to confirm whether this presentation really utilizes recycled MHC class I molecules, we treated DCs with primaquine, which blocks the membrane recycling pathway. DCs incubated in the presence of this drug could not present the 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, DCs were incubated with Hsp90-SL8C in the presence of chloroquine, a known inhibitor of acidification of endosomal compartments.

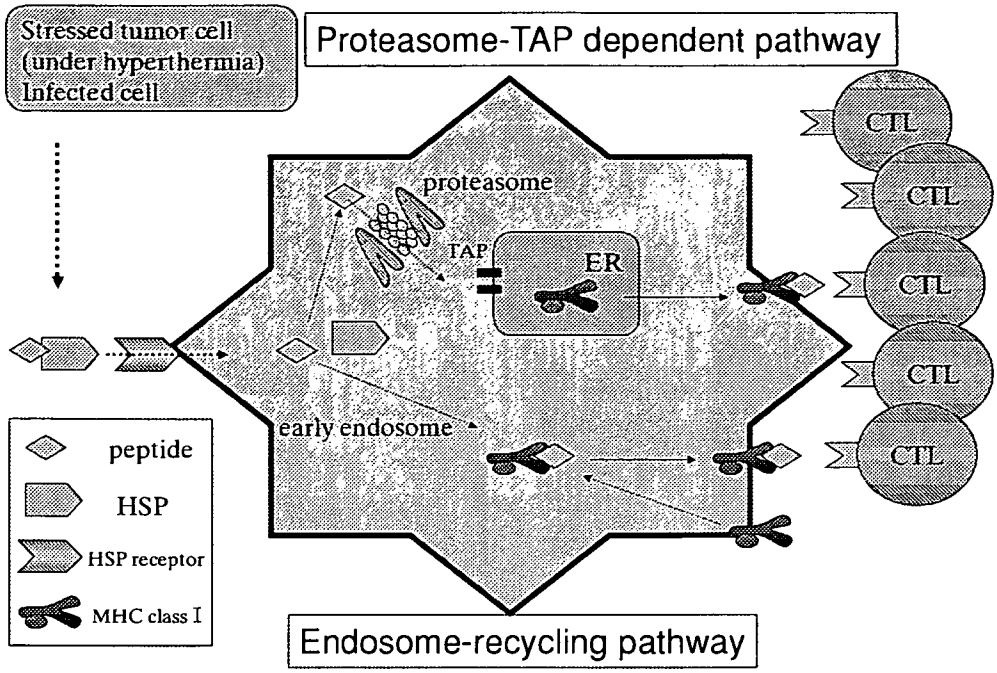

Fig. 3 **Possible pathway of Hsp90-antigen complex-mediated cross-presentation.** Internalized Hsp90-antigen complexes through receptor-mediated endocytosis follow 2 distinct MHC class I pathways. (1) Internalized Hsp90-antigen complexes are transferred to the cytosol and imported into the ER in a TAP-dependent 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.

Chloroquine strongly inhibited Hsp90-mediated presentation without affecting SL8 peptide presentation. Thus, acidification of endosomal compartments is necessary for processing of Hsp90-chaperoned precursor peptides (Fig. 3).

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 DCs, 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 APCs, including DCs, 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 OVA protein as a model antigen. We observed that the Hsp90-OVA antigen complex generated in vitro was very efficiently and selectively presented via the MHC class I pathway both in vitro and in vivo. Surprisingly and unexpectedly, we observed that the cross-presentation of Hsp90-OVA complexes was involved both TAP-dependent and -independent pathways, unlike the results of Hsp90-precursor peptide complex. These results provide a rationale for the development of novel vaccination strategies for cancer immunotherapy.

Hsp90-OVA complex is efficiently cross-presented by DCs.

We evaluated cross-presentation of the Hsp90-OVA protein complex. DCs 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 preferentially presented through MHC class I, but not class II 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, 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.

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[12]. 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.

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

The mechanism for antigen 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 DCs maintained mildly acidic pH in the endocytic compartment even after antigen uptake and could transport these antigens into the cytosol[21]. Chloroquine treatment inhibits the acidification of the endocytic compartments. Our data indicated that chloroquine treatment inhibited Hsp90-OVA presentation by DCs 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 DCs. However, a recent report showed that treatment with chlorquine or NH_4Cl enhanced the efficiency of cross-presentation[33]. These treatments accelerated export of exogenous soluble antigens from endocytic compartments to cytosol, thereby enhancing cross-presentation. The difference between

Fig. 4 **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.

our results and theirs was the method of uptake by DCs. 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 complete understanding of HSP-mediated immune regulation, and further studies will be required to elucidate the precise mechanism (Fig. 4).

Conclusion

Although HSPs 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 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 elucidate the mechanism of the driving force toward the $CD8^+$ T cell response mediated by HSPs. In addition, how endocytosed HSP-chaperoned antigens are translocated to be processed? These findings will clarify the impact of HSP as a danger signal in the etiologies of autoimmune diseases and tumor immunity.

References

1) Castelli, C., L. Rivoltini, F. Rini, F. Belli, A. Testori, M. Maio, V. Mazzaferro, J. Coppa, P.K. Srivastava, and G. Parmiani. 2004. Heat shock proteins: biological functions and clinical application as personalized vaccines for human cancer. *Cancer Immunol Immunother* 53:227-233.
2) Mazzaferro, V., J. Coppa, M.G. Carrabba, L. Rivoltini, M. Schiavo, E. Regalia, L. Mariani, T. Camerini, A. Marchiano, S. Andreola, R. Camerini, M. Corsi, J.J. Lewis, P.K. Srivastava, and G. Parmiani. 2003. Vaccination with autologous tumor-derived heat-shock protein gp96 after liver resection for metastatic colorectal cancer. *Clin Cancer Res* 9:3235-3245.
3) Rivoltini, L., C. Castelli, M. Carrabba, V. Mazzaferro, L. Pilla, V. Huber, J. Coppa, G. Gallino, C. Scheibenbogen, P. Squarcina, A. Cova, R. Camerini, J.J. Lewis, P.K. Srivastava, and G. Parmiani. 2003. Human tumor-derived heat shock protein 96 mediates in vitro activation and in vivo expansion of melanoma- and colon carcinoma-specific T cells. *J Immunol* 171:3467-3474.
4) Delneste, Y. 2004. Scavenger receptors and heat-shock protein-mediated antigen cross-presentation. *Biochem Soc Trans* 32:633-635.
5) Doody, A.D., J.T. Kovalchin, M.A. Mihalyo, A.T. Hagymasi, C.G. Drake, and A.J. Adler. 2004. Glycoprotein 96 can chaperone both MHC class I- and class II-restricted epitopes for in vivo presentation, but selectively primes $CD8^+$ T cell effector function. *J Immunol* 172:6087-6092.

6) Binder, R., D. Han, and P. Srivastava. 2000. CD91: a receptor for heat shock protein gp96. *Nature Immunol.* 1:151-155.
7) Delneste, Y., G. Magistrelli, J. Gauchat, J. Haeuw, J. Aubry, K. Nakamura, N. Kawakami-Honda, L. Goetsch, T. Sawamura, J. Bonnefoy, and P. Jeannin. 2002. Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity* 17:353-362.
8) Becker, T., F.U. Hartl, and F. Wieland. 2002. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. *J Cell Biol* 158:1277-1285.
9) Berwin, B., J.P. Hart, S. Rice, C. Gass, S.V. Pizzo, S.R. Post, and C.V. Nicchitta. 2003. Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. *Embo J* 22:6127-6136.
10) SenGupta, D., P.J. Norris, T.J. Suscovich, M. Hassan-Zahraee, H.F. Moffett, A. Trocha, R. Draenert, P.J. Goulder, R.J. Binder, D.L. Levey, B.D. Walker, P.K. Srivastava, and C. Brander. 2004. Heat shock protein-mediated cross-presentation of exogenous HIV antigen on HLA class I and class II. *J Immunol* 173:1987-1993.
11) Haug, M., L. Dannecker, C.P. Schepp, W.W. Kwok, D. Wernet, J.H. Buckner, H. Kalbacher, G.E. Dannecker, and U. Holzer. 2005. The heat shock protein Hsp70 enhances antigen-specific proliferation of human $CD4^+$ memory T cells. *Eur J Immunol* 35:3163-3172.
12) Ramirez, S.R., H. Singh-Jasuja, T. Warger, S. Braedel-Ruoff, N. Hilf, K. Wiemann, H.G. Rammensee, and H. Schild. 2005. Glycoprotein 96-activated dendritic cells induce a CD8-biased T cell response. *Cell Stress Chaperones* 10:221-229.
13) Udono, H., and P.K. Srivastava. 1994. Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90, and hsp70. *J Immunol* 152:5398-5403.
14) Kunisawa, J., and N. Shastri. 2006. Hsp90alpha chaperones large C-terminally extended proteolytic intermediates in the MHC class I antigen processing pathway. *Immunity* 24:523-534.
15) Basu, S., R.J. Binder, R. Suto, K.M. Anderson, and P.K. Srivastava. 2000. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int Immunol* 12:1539-1546.
16) Asea, A., M. Rehli, E. Kabingu, J.A. Boch, O. Bare, P.E. Auron, M.A. Stevenson, and S.K. Calderwood. 2002. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 277:15028-15034.
17) Vabulas, R.M., P. Ahmad-Nejad, S. Ghose, C.J. Kirschning, R.D. Issels, and H. Wagner. 2002. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *J Biol Chem* 277:15107-15112.
18) Basu, S., R.J. Binder, T. Ramalingam, and P.K. Srivastava. 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* 14:303-313.
19) Becker, T., F.U. Hartl, and F. Wieland. 2002. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. *J Cell Biol* 158:1277-1285.
20) Rodriguez, A., A. Regnault, M. Kleijmeer, P. Ricciardi-Castagnoli, and S. Amigorena. 1999. Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat Cell Biol* 1:362-368.
21) Hotta, C., H. Fujimaki, M. Yoshinari, M. Nakazawa, and M. Minami. 2006. The delivery of an antigen from the endocytic compartment into the cytosol for cross-presentation is restricted to early immature dendritic cells. *Immunology* 117:97-107.
22) Guermonprez, P., L. Saveanu, M. Kleijmeer, J. Davoust, P. Van Endert, and S. Amigorena. 2003. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* 425:397-402.
23) Houde, M., S. Bertholet, E. Gagnon, S. Brunet, G. Goyette, A. Laplante, M.F. Princiotta, P. Thibault, D. Sacks, and M. Desjardins. 2003. Phagosomes are competent organelles for antigen cross-presentation. *Nature* 425:402-406.
24) Ackerman, A.L., C. Kyritsis, R. Tampe, and P. Cresswell. 2003. Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc Natl Acad Sci U S A* 100:12889-12894.
25) Ackerman, A.L., C. Kyritsis, R. Tampe, and P. Cresswell. 2005. Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells. *Nat Immunol* 6:107-113.
26) Ackerman, A.L., A. Giodini, and P. Cresswell. 2006. A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. *Immunity* 25:607-617.
27) Gromme, M., F.G. Uytdehaag, H. Janssen, J. Calafat, R.S. van Binnendijk, M.J. Kenter, A. Tulp, D. Verwoerd, and J. Neefjes. 1999. Recycling MHC class I molecules and endosomal peptide loading. *Proc Natl Acad Sci U S A* 96:10326-10331.
28) Shen, L., L.J. Sigal, M. Boes, and K.L. Rock. 2004. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity* 21:155-165.
29) Suto, R., and P.K. Srivastava. 1995. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* 269:1585-1588.
30) Castellino, F., P.E. Boucher, K. Eichelberg, M. Mayhew, J.E. Rothman, A.N. Houghton, and R.N. Germain. 2000. Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. *J Exp Med* 191:1957-1964.
31) Moroi, Y., M. Mayhew, J. Trcka, M.H. Hoe, Y. Takechi, F.U. Hartl, J.E. Rothman, and A.N. Houghton. 2000. Induction of cellular immunity by immunization with novel hybrid peptides complexed to heat shock protein 70. *Proc Natl Acad Sci U S A* 97:3485-3490.
32) Kurotaki, T., Y. Tamura, G. Ueda, J. Oura, G. Kutomi, Y. Hirohashi, H. Sahara, T. Torigoe, H. Hiratsuka, H. Sunakawa, K. Hirata, and N. Sato. 2007. Efficient Cross-Presentation by Heat Shock Protein 90-Peptide Complex-Loaded Dendritic Cells via an Endosomal Pathway. *J Immunol* 179:1803-1813.
33) Accapezzato, D., V. Visco, V. Francavilla, C. Molette, T. Donato, M. Paroli, M.U. Mondelli, M. Doria, M.R. Torrisi, and V. Barnaba. 2005. Chloroquine enhances human $CD8^+$ T cell responses against soluble antigens in vivo. *J Exp Med* 202:817-828.

Efficient Cross-Presentation by Heat Shock Protein 90-Peptide Complex-Loaded Dendritic Cells via an Endosomal Pathway

Takehiro Kurotaki,*† Yasuaki Tamura,[1]* Gosei Ueda,*‡ Jun Oura,* Goro Kutomi,* Yoshihiko Hirohashi,* Hiroeki Sahara,* Toshihiko Torigoe,* Hiroyoshi Hiratsuka,‡ Hajime Sunakawa,‡ Koichi Hirata,† and Noriyuki Sato*

It is well-established that heat shock proteins (HSPs)-peptides complexes elicit antitumor responses in prophylactic and therapeutic immunization protocols. HSPs such as gp96 and Hsp70 have been demonstrated to undergo receptor-mediated uptake by APCs with subsequent representation of the HSP-associated peptides to MHC class I molecules on APCs, facilitating efficient cross-presentation. On the contrary, despite its abundant expression among HSPs in the cytosol, the role of Hsp90 for the cross-presentation remains unknown. We show here that exogenous Hsp90-peptide complexes can gain access to the MHC class I presentation pathway and cause cross-presentation by bone marrow-derived dendritic cells. Interestingly, this presentation is TAP independent, and followed chloroquine, leupeptin-sensitive, as well as cathepsin S-dependent endosomal pathways. In addition, we show that Hsp90-chaperoned precursor peptides are processed and transferred onto MHC class I molecules in the endosomal compartment. Furthermore, we demonstrate that immunization with Hsp90-peptide complexes induce Ag-specific CD8$^+$ T cell responses and strong antitumor immunity in vivo. These findings have significant implications for the design of T cell-based cancer immunotherapy. *The Journal of Immunology,* **2007, 179: 1803–1813.**

Heat shock proteins (HSPs)[2] are molecular chaperones that control the folding and prevent the aggregation of proteins. Recent studies have demonstrated that tumor-derived HSP, such as Hsp70, Hsp90, and gp96, initiate tumor-specific CTL responses and protective immunity (1). In this process, APCs internalize exogenously administered HSP with bound peptides by receptor-mediated endocytosis, resulting in Ag presentation via MHC class I molecules (2–5). Indeed, though a number of cellular receptors for HSPs have been described, including CD91 (6), CD40 (7), TLR2/4 (8), LOX-1 (9), and SR-A (10), as receptors for several kinds of HSPs (11), it is not clear which receptors are responsible for uptake and/or proinflammatory signaling. Tumor-bearing mice immunized with Hsp70 or gp96 isolated from the tumors or complexes of Hsp70 or gp96 reconstituted in vitro with known peptide Ags have been shown to mount a potent CD8$^+$ T cell response that can reduce or eliminate tumor progression (12–14).

In contrast, dendritic cells (DCs) have the capacity to take up, process, and present exogenous Ags in association with MHC class I molecules. This process is termed cross-presentation and the resulting CD8$^+$ T cell priming is referred to as cross-priming. It has been demonstrated that some exogenous Ags such as HSPs and particulated protein Ags gain access to the MHC class I-processing pathway and initiate CTL responses (1). This exogenous pathway is important for the development of CD8$^+$ CTL responses against tumors and infectious pathogens that do not have access to the classical MHC class I pathway. Administration of antigenic peptides in the context of purified HSPs induces potent CD8$^+$ T cell responses, indicating that HSP-peptide complexes can access the MHC class I endogenous Ag-presentation pathway (2, 13, 15–19). Thus, shuttling exogenous peptides into the endogenous pathway might be a specialized function of HSPs. However, the precise mechanism of HSP-mediated cross-presentation remains to be elucidated.

Considering the significance of HSP-mediated cross-presentation in vivo, the release of tumor cell contents, presumably including HSPs, and their uptake by APC would occur when tumor cells die either naturally, as a result of hypoxia, or because of therapeutic intervention (20–22). Given the important role of HSPs as a "danger signal," proposed by Matzinger and colleague (23), HSPs released into the extracellular milieu may act simultaneously as an Ag source due to their ability to chaperone peptides and as a maturation signal for dendritic cells, thereby inducing DCs to cross-present Ags to CD8$^+$ T cells.

Hsp90 is one of the 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 act as a danger signal, subsequently eliciting cell-specific immune responses. It has been demonstrated that the tumor-derived Hsp90-peptide complex elicits tumor-specific immunity (24). Moreover, Kunisawa and Shastri (25) have recently shown that cells generate large, C-terminally extended proteolytic intermediates that are associated with Hsp90. In this context, cell-derived Hsp90-C-terminally extended precursor peptide complexes could be released into the extracellular milieu, followed by uptake by APCs, when tumor cells are exposed to stress. At

*Department of Pathology, School of Medicine, Sapporo Medical University, Sapporo, Japan; †Department of Surgery, School of Medicine, Sapporo Medical University, Sapporo, Japan; and ‡Department of Oral and Maxillofacial Surgery, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan

Received for publication November 13, 2006. Accepted for publication May 11, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Yasuaki Tamura, Department of Pathology, School of Medicine, Sapporo Medical University, South 1, West 17, Chuo-ku, Sapporo 060-8556, Japan. E-mail address: ytamura@sapmed.ac.jp

[2] Abbreviations used in this paper: HSP, heat shock protein; DC, dendritic cell; BMDC, bone marrow-derived DC; VSV, vesicular stomatitis virus; α2M, α2 macroglobulin; SR-A, scavenger receptor A; ER, endoplasmic reticulum.

 0022-1767/07/$2.00

present, however, the Ag-processing pathway yielding the transfer of exogenous Hsp90-associated peptide Ags to MHC class I molecules is unknown.

In this study, we examined the roles of Hsp90 in MHC class I-restricted cross-presentation using bone marrow-derived DC (BMDC) as APCs. We show that Hsp90-peptide complexes reconstituted in vitro enter the endosomal pathways, which are chloroquine-, leupeptin-, and cathepsin S-sensitive. Furthermore, we show that chaperoned peptides are loaded onto endosomal (recycling) MHC class I molecules in the early endosome. Intriguingly, this presentation occurs within 30 min, indicating that very rapid and efficient processing might be achieved within BMDC. Moreover, we show that immunization with Hsp90-peptide complexes elicits very strong peptide-specific CTLs in vivo as well as therapeutic effects. Thus, we propose that Hsp90 acts as an excellent guide for cross-presentation of chaperoned Ags. Our data provide novel insights into the role of extracellular Hsp90-peptide complexes in cross-priming and peptide-based cancer immunotherapy.

Materials and Methods

Mice

C57BL/6 (H-2^b), B6C3F1 (H-$2^{b/k}$), and TAP1$^{-/-}$ mice were obtained from The Jackson Laboratory. HLA-A*2402/K^b transgenic mice were purchased from SLC Japan. All mice were kept in a specific pathogen-free mouse facility. Studies were performed according to institutional guidelines for animal use and care.

Cells

The mouse thymoma cell line EL4 and its E.G7 derivative (EL4 transfected with cDNA encoding OVA) were obtained from American Type Culture Collection (ATCC). N1 is an EL4 cell transfected with the nucleocapsid gene of vesicular stomatitis virus (VSV). An established CTL clone specific for the VSV8 epitope in the context of H-$2K^b$ was restimulated with N1 every 7 days. The B3Z cell is a $CD8^+$ T cell hybridoma specific for the $OVA_{258-265}$ epitope (SL8) in the context of H-$2K^b$. The KZO cell is a $CD4^+$ T cell hybridoma specific for the $OVA_{247-265}$ (PL19) in the context of I-A^k. These hybridomas were a gift from Dr. N. Shastri (University of California, Berkeley, CA). RMA-S-A*2402 cells were RMA-S transfected with the gene encoding HLA-A*2402 (provided by Dr. H. Takasu, Dainippon-Sumitomo Pharmaceutical, Osaka, Japan). TG-3 cells are a methylcholanthrene-induced fibrosarcoma derived from the HLA-A*2402 transgenic mouse. TG-3-2B cells were TG-3 transfected with the gene encoding human survivin-2B. Survivin-$2B_{80-88}$-specific CTLs were restimulated with survivin-$2B_{80-88}$ peptide-pulsed splenocytes every 7 days. BMDC were generated from the femurs and tibiae of C57BL/6 or HLA-A*2402/K^b transgenic mice. The bone marrow was flushed out, and the leukocytes were obtained and cultured in complete RPMI 1640 with 10% heat-inactivated FCS and 20 ng/ml GM-CSF (Endogen) for 5 days. On day 3, fresh medium with GM-CSF was added to the plates for the day 5 cultures.

Plasmid construction

FLAG-tagged human survivin-2B cDNA was amplified from HeLa cells by RT-PCR method and constructed in the plasmid pCDNA3.1$^+$ (Invitrogen Life Technologies). Primer pairs used for RT-PCR were 5′-CGGGAT CCATGGGTGCCCCGACGTTGCCC-3′ and 5′-CCGCTCGAGATCCA TGGCAGCCAGCTGCTC-3′ as forward and reverse primers, containing *Bam*HI and *Xho*I sites, respectively. The purified PCR product was digested with *Bam*HI and *Xho*I restriction enzymes, then ligated into digested pcDNA3.1$^+$ plasmid. The sequence of the cDNA was confirmed with ABI Genetic analyzer PRIM 3100 (PerkinElmer).

Proteins and Abs

Purified human Hsp90 and recombinant human Hsp72 were purchased from StressGen Biotechnologies. Chicken OVA was purchased from Calbiochem. BSA, fucoidin, and α2 macroglobulin (α2M) were obtained from Sigma-Aldrich. mAbs anti-H-$2K^b$ (clone AF6-88.5), anti-H-$2D^b$ (clone 28-14-8), and anti-HLA-DR (L243) were purchased from BD Pharmingen. mAb anti-MHC class I (W6/32) was purchased from ATCC. mAb anti-HLA-A24 (C7709A2.6) was provided by Dr. P. G. Coulie (Christian de Duve Institute of Cellular Pathology, University of Louvain, Brussels, Belgium). mAb anti-HLA-A31 was established in our laboratory. Organelles were detected by confocal laser microscopy with specific Abs against KDEL (clone 10C3; StressGen Technologies) for ER, Rho B (Santa Cruz Biotechnology) for endosomes, and CD107a (LAMP-1) (clone 1D4B; BD Pharmingen) for lysosomes. Each Ab was labeled with Alexa Fluor 594 (Molecular Probes). mAb 25D1.16 specific for the $K^b/OVA_{257-264}$ complexes was provided by Dr. R. Germain (National Institutes of Health, Bethesda, MD). Anti-mouse CD16/CD32 Fc-block was purchased from BD Pharmingen.

Generation of Hsp90-peptide/protein complex in vitro

The following peptides were used (underlined sequences represent the precise MHC class I-binding epitope): survivin-$2B_{80-88}$ peptide (AYACNT STL), survivin-$2B_{75-93}$ (GPGTVAYACNTSTLGGRGG) VSV 8 (RGYV YQGL), VSV-C (RGYVYQGLKSGNVSC), SL8 (SIINFEKL), and SL8C (SIINFEKLTEWTS). In vitro reconstitution was conducted as previously described (13). Hsp90 was mixed with a ^{125}I-labeled peptide in a 50:1 peptide to a protein molar ratio in 0.7 M NaCl containing sodium-phosphate buffer and heated at 45°C for 10 min then incubated at room temperature for 30 min. Hsp90 (10 μg) and OVA (10 μg) were mixed and incubated for 10 min at 45°C. The samples were then incubated for 30 min at room temperature. Free OVA was removed completely using a Microcon YM-100 (Millipore). In the case of Hsp72, peptides and Hsp72 were co-incubated at 37°C in sodium phosphate buffer containing 1 mM ADP and 1 mM $MgCl_2$. Samples were analyzed by SDS-PAGE and staining, followed by autoradiography of the stained gel.

Vaccination and induction of CTL

Each HLA-A*2402/K^b transgenic mouse was immunized s.c. at the base of the tail four times at 1-wk intervals, with Hsp90 (50 μg) alone, the Hsp90 (50 μg)-survivin-$2B_{80-88}$ peptide (AYACNTSTL) (50 μg) complex, survivin-$2B_{80-88}$ peptide (50 μg) with IFA or CFA individually. One week after the last immunization, spleen cells were removed, cultured in vitro with irradiated (100 Gy) and survivin-$2B_{80-88}$ peptide-pulsed spleen cells for 5 days. Subsequently, the generation of survivin-$2B_{80-88}$ peptide-specific CTLs was evaluated in a ^{51}Cr-release assay. The specificity of CTLs induced in an individual HLA-A*2402/K^b transgenic mouse was evaluated using RMA-S/A*2402 cells as targets in the presence or absence of the survivin-$2B_{80-88}$ peptide.

^{51}Cr-release assay

The cytolytic activity of the induced CTL was determined by a standard 4 h-^{51}Cr-release assay described earlier (19). To determine the MHC class I restriction in the cytotoxic assay, indicated amount of mAbs against HLA class I (W6/32), HLA-A24, HLA-A31, and HLA class II (L231) were added to each well. In the case of VSV or OVA system, 10 μg/ml mAbs against H-$2K^b$ and H-$2D^b$ were added to the each well.

Transplantation of tumor cells and immunotherapy

TG3-2B cells (5×10^5) were intradermally transplanted into the right flank in HLA-A*2402/K^b transgenic mice on day 0. When average tumor diameter reached 5 mm, the mice were then treated with Hsp90 (50 μg) alone, the Hsp90 (50 μg)-survivin-$2B_{80-88}$ peptide (50 μg) complex, or survivin-$2B_{80-88}$ peptide (50 μg) emulsified in IFA via s.c. administration at the nape of the neck twice each week for 2 wk (on days 9, 13, 16, and 20). Control groups of mice were immunized with PBS. Tumor growth was recorded twice each week. Average diameters of the two axes were plotted so that therapeutic effects could be compared among the groups. Average tumor diameters on day 29 were statistically analyzed using the Mann-Whitney *U* test. In addition, mouse survival was monitored every other day. Statistical analyses for evaluating the survival advantages were performed using log-rank analysis. All the experiments were performed with 10 mice/group.

ELISPOT assay

The specificity of CTLs for the survivin-$2B_{80-88}$ peptide was also evaluated by IFN-γ ELISPOT assay. Splenic $CD8^+$ T cells were isolated from mice immunized with Hsp90-survivin-$2B_{80-88}$ peptide, which were cultured for 5 days described above, with MACS (Miltenyi Biotec) using an anti-mouse CD8a mAb coupled with magnetic microbeads according to the manufacturer's instructions. As target cells, RMA-S/A*2402 cells were cultured overnight at 26°C in RPMI 1640 supplemented with 10% FBS, 2.5 μg/ml β_2-microglobulin, 100 μg/ml survivin-$2B_{80-88}$ peptide, an irrelevant CMV peptide (QYDPVAALF), or without any peptide. Ninety-six-well ELISPOT plates (BD Biosciences) were coated with 5.0 μg/ml rat

anti-mouse IFN-γ mAb and subsequently blocked with RPMI 1640 supplemented with 10% FBS for 2 h at room temperature. Then, 5×10^3 CTLs and 1×10^5 each target cells were added to the wells and cultured for 12 h at 37°C in RPMI 1640 in 10% FBS. The plates were then washed extensively and incubated with a 2.0 μg/ml biotinylated anti-mouse IFN-γ mAb, followed by pulsing with 0.5 μg/ml streptavidin-HRP. Positive spots were developed by adding 100 μl/well AEC Substrate Solution (BD Biosciences) and were counted using a Vision ELISPOT reader (Carl Zeiss).

Production of retrovirus and virus infection

The HLA-A*2402 cDNA was inserted into *Bam*HI and *Not*I sites of the pMXs-puro retrovirus expression vector (26) (gift from Prof. T. Kitamura, University of Tokyo, Tokyo, Japan). High-titer retrovirus carrying HLA-A*2402 was produced in a transient retrovirus-packaging cell line PLAT-E (27) (gift from Prof. T. Kitamura). Briefly, PLAT-E cells were transfected with 5 μg of retrovirus vector plasmid with the FuGene HD Transfection Reagent (Roche Molecular Diagnostics). At 48 h after transfection, the supernatant was harvested as viral stock solution. For infection, immature BMDCs from $TAP^{-/-}$ mice were incubated for 6 h with 6 ml of virus stock solution in the presence of 8 μg/ml hexadimethrine bromide (Sigma-Aldrich). Twenty-four hours postinfection, the mouse BMDCs were used for assays. The expression of HLA-A24 was confirmed by flow cytometry using an anti-HLA-A24 mAb (C7709A2.6) and 20–30% of DCs were HLA-A24 positive.

In vitro cross-presentation assay

Immature BMDCs (1×10^4) from HLA-A*2402/K^b transgenic mice or HLA-A*2402-transduced BMDCs (1×10^4) derived from $TAP^{-/-}$ mice were pulsed with an Hsp90 (10 μg/ml)-survivin-$2B_{75-93}$ precursor peptide (100 ng/ml) complex generated in vitro, Hsp90 alone (10 μg/ml), survivin-$2B_{80-88}$ peptide (100 ng/ml), or survivin-$2B_{75-93}$ precursor peptide (100 ng/ml), and survivin-$2B_{80-88}$-specific CTLs (1×10^5) were added to the cultures. In VSV or OVA system, immature BMDCs (1×10^4) from C57BL/6 and $TAP^{-/-}$ mice were pulsed with an Hsp90 (10 μg/ml)-peptide (10 μg/ml) or OVA protein (10 μg/ml) complex generated in vitro, and peptide-specific CTLs (1×10^5) were added to the cultures. The assay was conducted in 200-μl volume in 96-well plates with AIM-V (Invitrogen Life Technologies) at 37°C for 20 h. Culture supernatants were harvested and tested for the presence of IFN-γ release by ELISA (Cytimmune Sciences). In the case of OVA system, SL8/K^b-specific B3Z or PL19/A^k-specific KZO responses were measured as the β-galactosidase activity induced upon ligand recognition. The B3Z (1×10^5) or KZO (1×10^5) and Ag-loaded BMDCs (5×10^4) were added to each well and cultured overnight. The β-galactosidase activity was measured at the absorbance at 595 nm of the cleavage product of chlorophenol red β-pyranoside.

Immunocytological localization of exogenous Hsp90

Hsp90 was conjugated with Alexa Fluor 488 (Molecular Probes) according to the manufacturer's instructions. Immature BMDCs were pulsed with the Alexa Fluor 488-labeled Hsp90 (20 μg/ml)-SL8C peptide (20 μg/ml) complex for 2 h. After incubation, cells were fixed with ice-cold acetone for 1 min, and then stained with an anti-Rho B Ab for detecting early endosomes, anti-KDEL mAb for ER, and anti-LAMP1 for late endosomes and lysosomes followed by Alexa Fluor 594-conjugated goat anti-rabbit IgG or anti-mouse IgG and visualized using a Bio-Rad MRC1024ES laser confocal scanning microscopy system (Bio-Rad). For detecting the intracellular localization of recycling MHC class I molecules and SL8-K^b complexes, the DCs were incubated with anti-mouse CD16/CD32 Fc-block to block nonspecific staining, and then costained with an Alexa Fluor 488-labeled Hsp90-SL8C peptide complex and Alexa Fluor 594-labeled anti-H-$2K^b$ mAb (clone AF6-88.5) or Alexa Fluor 488-labeled 25D1.16 mAb and anti-organelle Abs conjugated with Alexa Fluor 594 and visualized by confocal laser microscopy.

Detection of fluorescent Hsp90-peptide complexes association with BMDCs and competition assay

BMDCs (1×10^5) were preincubated with Hsp90, BSA, human α2M (Sigma-Aldrich), or fucoidin (Sigma-Aldrich) at indicated concentrations (25, 50, 100 μg) for 10 min at 4°C, then pulsed with the Alexa Fluor 488-labeled Hsp90 (5 μg)-SL8 peptide (10 μg) complex for 10 min at 4°C. The BMDCs were then washed twice with ice-cold PBS, fixed with ice-cold acetone for 1 min, and analyzed by flow cytometry and confocal laser microscopy.

Inhibition studies

For most inhibition studies, immature BMDCs (10^5 cells/well) were first incubated in 0.1 ml of each drug for 2 h. Then Hsp90 (10 μg/ml)-SL8C (10 μg/ml) complex, SL8C (10 μg/ml) or OVA protein (40 μg/ml) of Ag was added to the wells (0.2 ml final volume) at the final concentration indicated in the continuous presence of inhibitors for 2 h. BMDCs were then washed three times and fixed with 0.05% glutaraldehyde (Sigma-Aldrich). Fixation was stopped by addition of 2 M L-lysine (Sigma-Aldrich) and cells were washed twice in PBS. Thereafter, SL8/K^b-specific B3Z or PL19/A^k-specific KZO were added to each well and cultured overnight. The β-galactosidase activity was measured at the absorbance at 595 nm. The inhibitors used were primaquine, chloroquine, lactacystin, leupeptin, and pepstatin (all obtained from Sigma-Aldrich except for primaquine (ICN Biomedicals)). LLnL, cathepsin S inhibitor (Z-FL-COCHO), cathepsin B inhibitor (Ac-Leu-Val-lysinal), and cathepsin L inhibitor (Z-FF-FMK) were purchased from Merck Biosciences.

Results

Efficient induction of tumor peptide-specific CTL by immunizing Hsp90-peptide complex

We previously reported that survivin and its splicing variant survivin-2B are expressed abundantly in various types of tumor tissues and are suitable as target Ags for tumor immunotherapy. Subsequently, we identified an HLA-A24-restricted antigenic peptide, survivin-$2B_{80-88}$ (AYACNTSTL) recognized by $CD8^+$ CTLs (28, 29). On the basis of these observations, we have started a phase I clinical study of survivin-$2B_{80-88}$ peptide vaccination for patients with advanced colorectal cancer. To establish an effective cancer vaccine, the development of an effective and safe adjuvant remains a high priority. Therefore, we examined whether HSPs could be a good candidate for a cancer vaccine adjuvant. First, we confirmed that the two major HSPs, Hsp70 and Hsp90, but not the control protein transferrin, were made complexed with survivin-$2B_{80-88}$ peptides in vitro (Fig. 1*A*). Next, we tested the ability of Hsp70 and Hsp90 to generate CTL responses against associated peptides. HLA-A*2402/K^b transgenic mice are a well-established model for studying HLA-A*2402-restricted CTL epitopes and vaccine development (30). These mice contain transgenic chimeric human α1 and α2 domains of HLA-A*2402 and mouse α3 transmembrane and cytoplasmic domains of H-$2K^b$, which increase the level of T cell responsiveness. We immunized these transgenic mice with the Hsp70-survivin-$2B_{80-88}$ peptide complex or Hsp90-survivin-$2B_{80-88}$ peptide complex and examined the induction of peptide-specific CTL responses. As shown in Fig. 1*B*, spleen cells of mice immunized with the Hsp90-survivin-$2B_{80-88}$ peptide complex showed significant cytotoxicity against survivin-$2B_{80-88}$-coated RMA-S-A*2402 cells, but not survivin-$2B_{80-88}$-noncoated RMA-S-A*2402 cells. This cytotoxic activity was almost same as from mice immunized with survivin-$2B_{80-88}$ emulsified in CFA (Fig. 1*G*). In contrast, spleen cells of mice immunized with the Hsp70-survivin-$2B_{80-88}$ peptide complex (Fig. 1*D*), Hsp90 alone (Fig. 1*C*), Hsp70 alone (Fig. 1*E*) or survivin-2B emulsified in IFA (Fig. 1*F*) did not show much cytotoxicity against survivin-$2B_{80-88}$-coated RMA-S-A*2402. This cytotoxicity was significantly blocked by pretreatment of target cells with an anti-human MHC class I mAb W6/32 and an anti HLA-A24 mAb, but not with an anti-HLA-A31 mAb (Fig. 1*H*). Similarly, pretreatment of effector cells with anti-CD8, but not with anti-CD4 mAb, significantly blocked the cytotoxicity against survivin-$2B_{80-88}$-coated RMA-S-A*2402 cells (data not shown). Furthermore, to determine whether this cytotoxic function was related to the frequency of peptide-specific T cells, ELISPOT assay was performed using splenocytes from each immunized mouse. Purified $CD8^+$ T cells from splenocytes after 5 days in vitro stimulated with survivin-$2B_{80-88}$ were cocultured for 12 h with RMA-S/A*2402cells alone, RMA-S/A*2402 pulsed with survivin-$2B_{80-88}$, or those pulsed with an

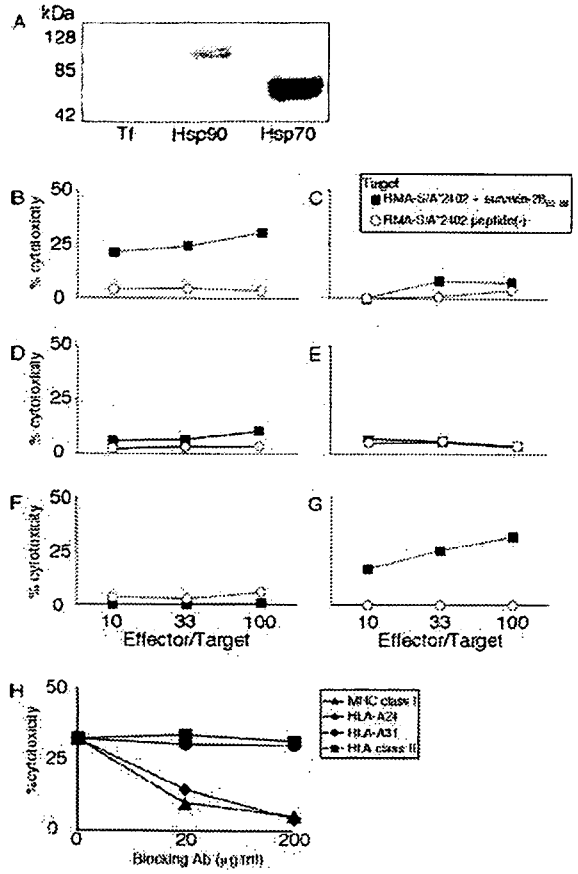

FIGURE 1. Efficient induction of peptide-specific CTL response in mice vaccinated with Hsp90-peptide complexes. *A*, Hsp90, Hsp70, and transferrin (Tf) were mixed with a ^{125}I-labeled peptide in a 50:1 peptide protein ratio in sodium-phosphate buffer. Samples were analyzed by SDS-PAGE and staining (data not shown), followed by autoradiography of the stained gel. Hsp90 and Hsp70 bound the survivin-2B$_{80-88}$ peptide efficiently, but not transferrin. *B–G*, HLA-A*2402/K^b-transgenic mice were immunized s.c. four times with the Hsp90-survivin-2B$_{80-88}$ peptide complex (*B*), Hsp90 alone (*C*), Hsp70-survivin-2B$_{80-88}$ peptide complex (*D*), Hsp70 alone (*E*), and survivin-2B$_{80-88}$ peptide emulsified with IFA (*F*) or CFA (*G*) individually. Spleen cells were removed 1 wk after the last immunization, cultured for 5 days with survivin-2B$_{80-88}$ peptides, and tested for cytotoxicity. Each line represents the specific lysis of target cells by spleen cells from one individual mouse. Target cells were RMA-S/A*2402 cells pulsed with the survivin-2B$_{80-88}$ peptide (■) or without the peptide (□). *H*, Peptide-specific cytotoxicity was induced in MHC class I-restricted fashion. Indicated amount of mAbs against HLA class I (W6/32), HLA-A24 (C7709A2.6), HLA-A31, and HLA class II (L231) were added to each well.

irrelevant CMVpp65-derived peptide (QYDPVAALF). The number of IFN-γ ELISPOTs produced by 5 × 10^3 CD8$^+$ T cells against 1 × 10^5 RMA-S/A*2402 cells are shown in Fig. 2. CD8$^+$ T cells efficiently produced IFN-γ ELISPOTs in response to RMA-S/*A2402 cells pulsed with survivin-2B$_{80-88}$ peptide, but not in response to RMA-S/A*2402 cells alone or RMA-S*A2402 cells pulsed with the irrelevant CMVpp65-derived peptide (QYDPVAALF). These findings confirmed the fact that the CTLs were specific for survivin2B$_{80-88}$. Thus, we demonstrated that Hsp90 was a fairly good adjuvant for a T cell-mediated antitumor vaccine.

Cross-presentation of Hsp90-chaperoned peptide in the context of MHC class I by BMDCs

Next, we tested whether in vitro reconstituted Hsp90-peptide complexes were taken up and associated peptides were presented in the

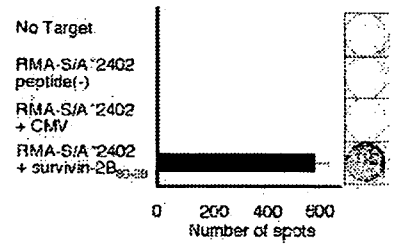

FIGURE 2. Immunization with Hsp90-Ag peptide complex generated CTLs recognizing chaperoned peptide. Purified splenic CD8$^+$ T cells from Hsp90-survivin-2B$_{80-88}$ peptide-immunized mice were stimulated with the survivin-2B$_{80-88}$ peptide in vitro. Responding CTLs were tested for IFN-γ ELISPOTs in response to RMA-S/A*2402 cells without peptide pulsing, pulsed with an irrelevant CMV peptide, or pulsed with the survivin-2B$_{80-88}$ peptide. The number of ELISPOTs produced by 5 × 10^3 CTLs in response to 1 × 10^5 RMA-S/A*2402 cells is shown.

context of MHC class I molecules by BMDCs. To monitor the MHC class I Ag-processing pathway, we used Hsp90 reconstituted in vitro with a precursor peptide of survivin2B$_{80-88}$, survivin-2B$_{75-93}$ (19 mer). The Hsp90-survivin-2B$_{75-93}$ precursor peptide complex was cocultured with BMDCs for 2 h and fixed, followed by incubation with a survivin-2B$_{80-88}$-specific CTL clone. The culture supernatant was assayed for the production of IFN-γ. As shown in Fig. 3, the Hsp90-survivin-2B$_{75-93}$ precursor peptide complex was processed and presented by HLA-A*2402, and consequently recognized by the survivin-2B$_{80-88}$-specific CTL clone but not Hsp90 or the survivin-2B$_{75-93}$ precursor peptide alone. This phenomenon was also observed by the standard ^{51}Cr-release assay (data not shown). These data suggested that the Hsp90-chaperoned precursor peptide was processed to an epitope within the cells with subsequent access to the MHC class I pathway, a process known as cross-presentation.

Vaccination of mice with Hsp90-peptide complex promotes antitumor effect

We then examined the efficacy of the Hsp90-based immunotherapy using the human tumor Ag survivin-2B as a surrogate Ag. To assess whether cross-presentation of the Hsp90-survivin-2B$_{80-88}$ complexes elicits antitumor effects in vivo, we developed TG3 with surrogate Ag human survivin-2B, TG3-2B. Although, the TG3-2B cell line is an artificial tumor model, we thought that it was necessary to examine whether the in vitro cross-presentation

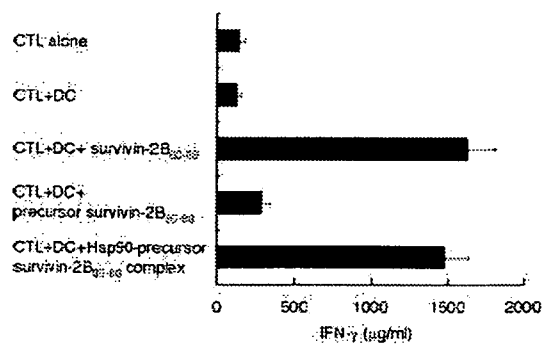

FIGURE 3. Cross-presentation of Hsp90-chaperoned peptide by BMDCs. Survivin-2B$_{80-88}$, precursor survivin-2B$_{80-88}$ (19 mer), or Hsp90-precursor survivin-2B$_{80-88}$ complex was loaded to HLA-A*2402/K^b-transgenic mouse-derived BMDCs for 2 h and a survivin-2B$_{80-88}$-specific CTL clone was added. IFN-γ in the culture supernatant was measured by ELISA.

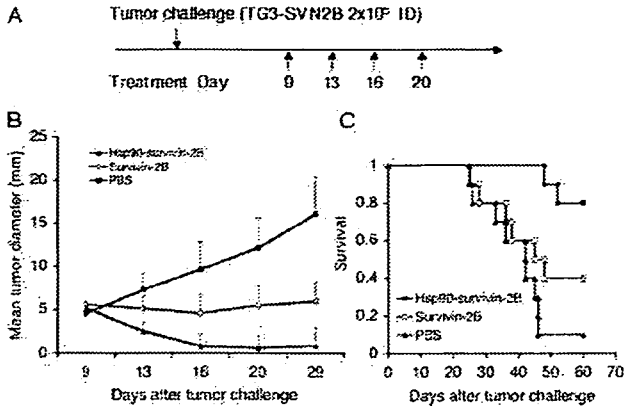

FIGURE 4. Hsp90-tumor Ag peptide complex induces strong antitumor effect. *A*, The protocol for immunotherapy is shown. *B*, A total of 5×10^5 TG-3-2B cells were first injected intradermally into HLA-A*2402/K^b mice (10 animals/group). When mean tumor diameter reached 5 mm, mice were given the treatment with the Hsp90 (50 μg)-survivin-2B$_{80-88}$ (50 μg) complex, survivin-2B$_{80-88}$ (50 μg) emulsified with IFA alone or PBS twice a week. *C*, The remaining 10 mice in each group were observed for survival.

of Hsp90-survivin-2B complexes represents the survivin-2B-specific antitumor effects in vivo. TG3-2B cells (2×10^5) were inoculated into the right hind limbs of HLA-A*2402/K^b transgenic mice and allowed to grow for 10 days (to around 5 mm in diameter). As shown in Fig. 4*A*, on days 9, 13, 16, and 20, mice were administered PBS, the survivin-2B$_{80-88}$ peptide (50 μg), or the Hsp90 (50 μg)-survivin-2B$_{80-88}$ (50 μg) complex. As shown in Fig. 4*B*, vaccination with Hsp90-survivin-2B$_{80-88}$ significantly inhibited the growth of tumors in comparison to control vaccinations with survivin-2B$_{80-88}$ or PBS, (vs survivin-2B, $p = 0.034$, vs PBS, $p = 0.0007$). We also evaluated the effects of the Hsp90-survivin-2B peptide complex in providing a survival benefit. As shown in Fig. 4*C*, the results indicated that treatment with the Hsp90-survivin-2B peptide complex significantly increased the median survival time compared with the control mice (vs survivin-2B, $p = 0.016$, vs PBS, $p = 0.001$). Of note, 8 of 10 animals of the Hsp90-survivin-2B$_{80-88}$ complex-treated groups rejected the established tumors. Taken together, these results showed not only that the Hsp90-peptide complex induced strong CTL responses to the chaperoned peptide but also that these responses were sufficiently strong to generate therapeutic antitumor effects.

TAP-independent presentation of Hsp90-bound peptide in the context of MHC class I by BMDCs

To generalize the Hsp90-mediated cross-presentation, we used a well-characterized antigenic system, the VSV derived H-2K^b-restricted VSV8 dominant Ag. To test the peptide-binding capacity of purified human Hsp90, we used synthetic peptides VSV8 and a C-terminal extended version of VSV8, VSV-C (15 mer). First, we confirmed the in vitro generation of the Hsp90-VSV-C peptide complex (data not shown). Next, we tested whether in vitro-reconstituted Hsp90-VSV-C complexes were taken up and associated peptides were presented in the context of MHC class I molecules by immature BMDCs. The Hsp90-VSV-C peptide complex was

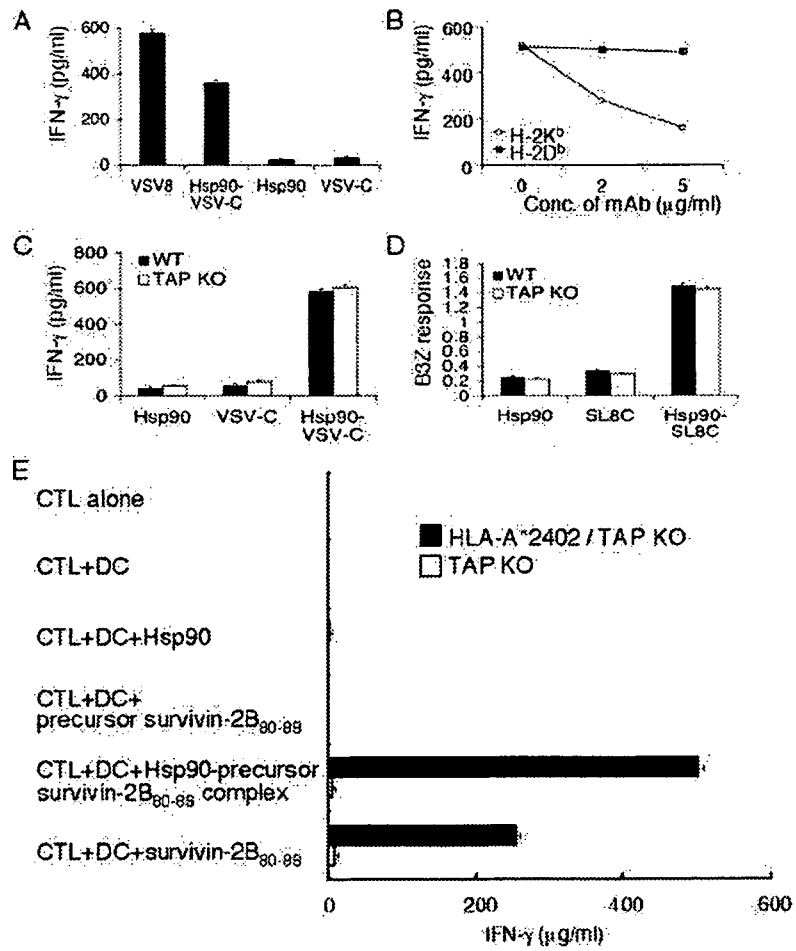

FIGURE 5. Precursor peptides chaperoned by Hsp90 are cross-presented via a TAP-independent pathway. *A*, VSV8, the Hsp90-VSV-C complex, Hsp90, or VSV-C was loaded to BMDCs for 2 h and a VSV8-specific CTL clone was added. IFN-γ in the culture supernatant was measured by ELISA. *B*, The anti-H-2K^b mAb, but not the H-2D^b mAb, inhibited cytotoxicity mediated by the VSV-8-specific CTL clone. *C*, BMDCs from the TAP$^{-/-}$ mouse could also process and present Hsp90-chaperoned VSV-C peptides efficiently as compared with BMDCs from the wild-type mouse. *D*, SL8 precursor peptide, SL8C was also processed and cross-presented by BMDCs via a TAP-independent pathway. *E*, HLA-A*2402-transduced TAP$^{-/-}$ BMDCs were able to cross-present Hsp90-chaperoned precursor survivin-2B$_{80-88}$ peptides in association with HLA-A*2402 molecules.

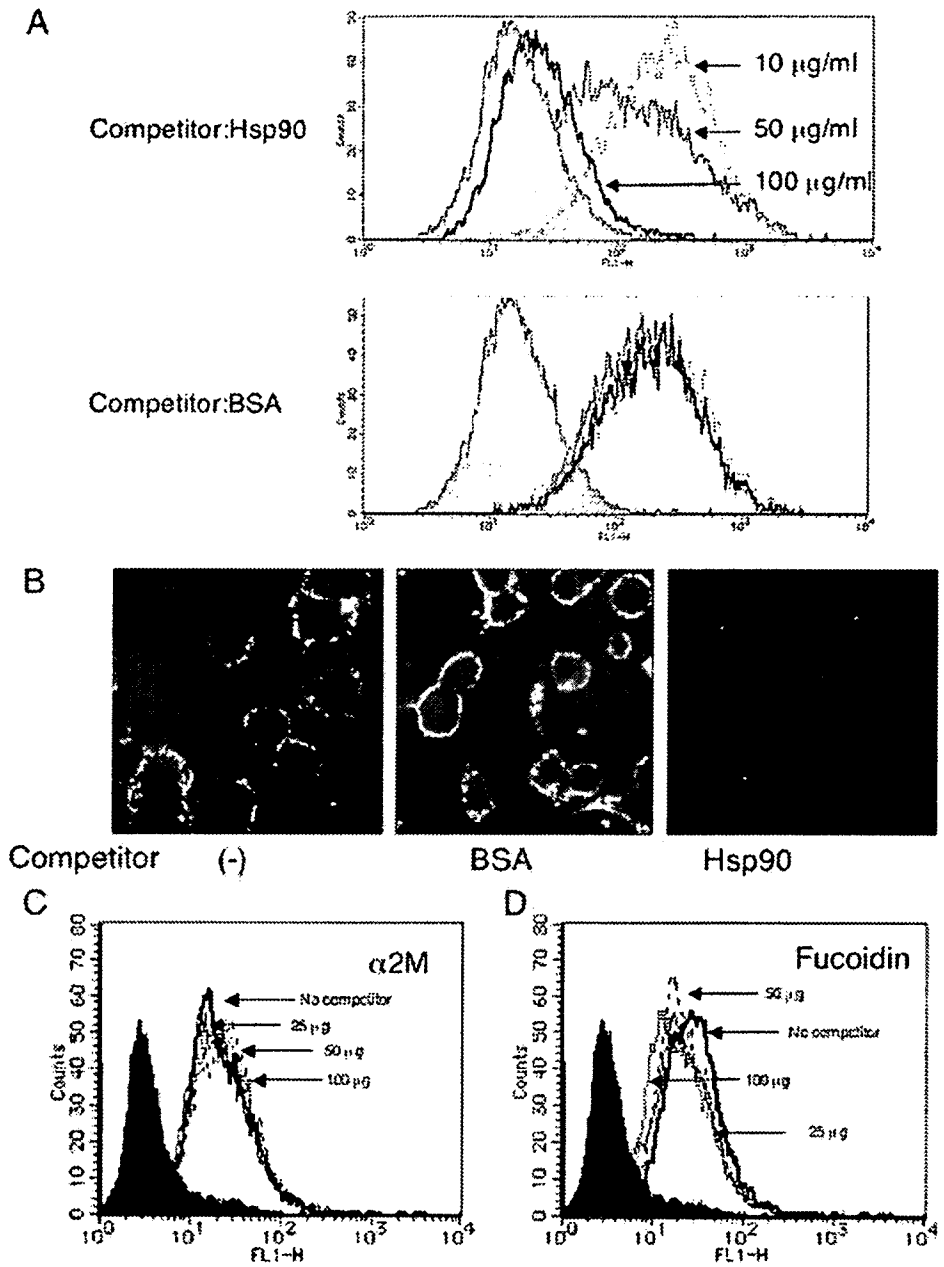

FIGURE 6. The Hsp90-peptide complex binds to immature BMDCs in a receptor-dependent fashion. *A*, BMDCs were incubated with the Alexa 488-labeled Hsp90-SL8C complex at 4°C to exclude endocytosis. Specific binding of the Hsp90-SL8C complex was observed and could be competed for by a 10-fold excess of unlabeled Hsp90. No inhibition was observed using an excess of up to 100-fold of BSA. *B*, BMDCs were incubated with unlabeled 50 μg/ml Hsp90 or 50 μg/ml BSA for 1 h on ice, then pulsed with 5 μg/ml Alexa 488-labeled Hsp90-SL8C complexes to DCs on ice for 1 h, washed with PBS, followed by acetone fixation and processing for confocal microscopy. *C* and *D*, The binding of the Hsp90-SL8C peptide complex to BMDCs was not inhibited by either the CD91 ligand α2M or the SR-A ligand fucoidin.

cocultured with immature BMDCs for 2 h and fixed, followed by incubation with a VSV8-specific CTL clone. The culture supernatant was assayed for the production of IFN-γ. As shown in Fig. 5*A*, the Hsp90-VSV-C peptide complex was processed and presented by H-2K^b, and consequently recognized by the VSV8-specific CTL clone but not Hsp90 or VSV-C alone. In the presence of an anti-H-2K^b mAb but not an anti-H-2D^b mAb, during the presentation assay, the presentation of VSV8 to the specific CTL clone was clearly abolished (Fig. 5*B*). These data suggested that Hsp90 bound VSV-C peptide was processed to VSV8 within the cells with subsequent access to the MHC class I pathway.

Next, we investigated whether the Hsp90-mediated MHC class I pathway required functional TAP molecules. To test this, we used immature BMDCs derived from the TAP1$^{-/-}$ mouse. Surprisingly, BMDCs from the TAP1$^{-/-}$ mouse could also process and present the Hsp90-bound VSV-C peptide as efficiently as BMDCs from the wild-type mouse (Fig. 5*C*). Taking advantage of the use of T cell hybridoma B3Z, we also tested another well-characterized H-2K^b-restricted OVA$_{257-264}$ Ag system. Hsp90 reconstituted in vitro with SL8C peptide (13mer, C-terminal extended version of SL8 (OVA$_{257-264}$)) was cocultured with BMDCs for 2 h, followed by incubation with an SL8-specific B3Z T cell hybridoma. As shown in Fig. 5*D*, the Hsp90-SL8C peptide complex but not Hsp90 or SL8C alone was processed and presented by H-2K^b, and recognized consequently by the B3Z T cell hybridoma in a TAP-independent manner.

Furthermore, we have confirmed that Hsp90-survivin-2B$_{75-93}$ precursor peptide complex was processed and presented by TAP$^{-/-}$ mice-derived BMDCs, which were retrovirally transduced with HLA-A*2402 cDNA, and consequently recognized by the survivin-2B$_{80-88}$-specific CTL clone but not Hsp90 or the survivin-2B$_{75-93}$ precursor peptide alone (Fig. 5*E*). These data demonstrated that a TAP-independent pathway was used for Hsp90-mediated MHC class I presentation.

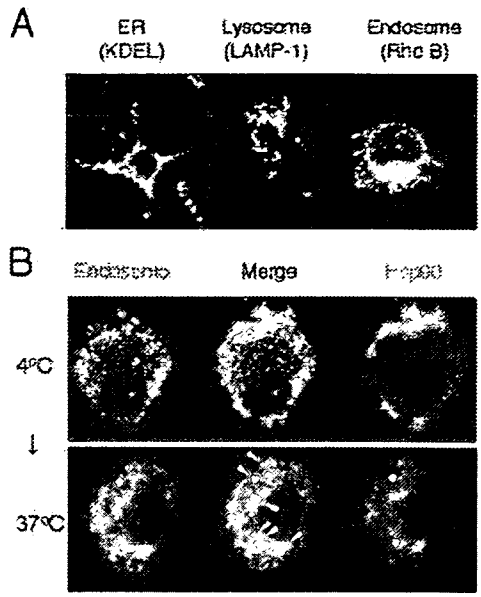

FIGURE 7. Intracellular localization of the Hsp90-SL8C peptide complex taken up by receptor-mediated endocytosis. *A*, Immature BMDCs were pulsed with Alexa 488-labeled Hsp90-SL8C complex for 2 h. After incubation, cells were fixed with cold acetone, stained with an anti-Rho B Ab (for early endosomes), anti-KDEL mAb (for ER), and anti-LAMP1 (for late endosomes and lysosomes) followed by Alexa 594-conjugated goat anti-rabbit IgG or anti-mouse IgG and visualized by laser confocal microscopy. *B*, Internalization of the Hsp90-peptide complex occurred via temperature-dependent endocytosis. BMDCs were treated either 4°C or at 37°C with 20 μg/ml Alexa 488-labeled Hsp90-SL8C complex for 10 min, washed with PBS, and fixed, then stained with the anti-Rho B Ab, followed by Alexa 594-conjugated goat anti-rabbit IgG and analyzed by laser confocal microscopy.

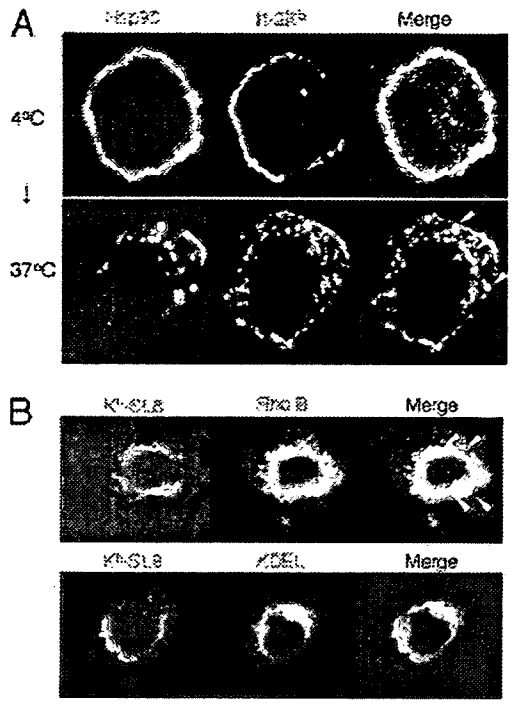

FIGURE 8. The Hsp90-peptide complex traffics to an endosome, where the precursor peptide might be processed, followed by the formation of a peptide-MHC class I complex. *A*, BMDCs were treated at either 4°C or at 37°C with the 20 μg/ml Alexa 488-labeled Hsp90-SL8C complex for 10 min, washed with PBS, and fixed, then stained using Alexa 594-conjugated anti-H-2K^b mAb. *B*, BMDCs were incubated with Hsp90-SL8C peptide complexes for 1 h, then fixed with ice-cold acetone for 1 min. DCs were incubated with mAb 2.4G2 to block FcR and then costained with Alexa 488-conjugated mAb 25D1.16 (for H-2K^b-SL8 complex) and Alexa 594-conjugated anti-Rho B Ab or anti-KDEL mAb.

Hsp90-peptide complex interacts with bone marrow-derived immature BMDCs in a receptor-dependent fashion

Recent experiments demonstrated that HSPs are able to interact specifically with macrophages, DC, and B cells. To test the specific binding of Hsp90-peptide complex to immature BMDCs, we incubated BMDCs with the Alexa 488-labeled Hsp90-SL8C complex at 4°C to exclude endocytosis. Using FACS analysis, we observed specific binding of Hsp90-peptide complexes to the cell surface that could be competed for by unlabeled Hsp90, but not by BSA (Fig. 6*A*). A 10-fold excess of unlabeled Hsp90 significantly inhibited the binding of Alexa 488-labeled Hsp90-SL8C complexes to BMDCs. No inhibition was observed using an excess of up to 100-fold of BSA. We then analyzed the competition experiments using the laser confocal microscopy. We have confirmed that after 1 h culture at 4°C, a temperature which blocks internalization, the Alexa 488-labeled Hsp90-SL8C complexes were found on the cell surface of immature BMDCs. Next, immature DCs were incubated with Alexa 488-labeled Hsp90-SL8C complexes, alone or in the presence of a 10-fold excess of unlabeled Hsp90 or BSA. As shown in Fig. 6*B*, competition with unlabeled Hsp90 significantly reduced the cell surface binding, whereas unlabeled BSA did not affect the binding as compared with the labeled Hsp90-peptide complex alone. These data demonstrated the presence of a specific receptor for Hsp90 that was expressed on immature BMDCs. Recently, CD91 (the α2M receptor), LOX-1, and scavenger receptor class-A (SR-A) were identified as the HSP receptors of APCs. Therefore, we have done the competition assay using α2M and fucoidin, which are known for the ligands of HSP receptors CD91 (for α2M), LOX-1, and SR-A (for fucoidin). The results showed that the twenty-fold concentration of either α2 macroglobulin (Fig. 6*C*) or fucoidin (Fig. 6*D*) was not able to compete the cell surface binding of Alexa Fluor 488-labeled Hsp90-SL8 complex. Therefore, we concluded that Hsp90 receptor was different from CD91 and scavenger receptors such as LOX-1 or SR-A in our experiments.

Endocytosed Hsp90-peptide complexes localize in the early endosome

Next, we were interested in addressing which compartments were involved in this processing and presentation of Hsp90-chaperoned precursor peptides within the BMDCs. We labeled human Hsp90-SL8C with Alexa Fluor 488. Labeled Hsp90-SL8C peptide complexes were then incubated with BMDCs for several time periods. After extensive washing to remove unbound proteins, cells were fixed with cold acetone and costained with an Ab against an organelle marker labeled with Alexa Fluor 594. Analysis by laser confocal microscopy of staining with the organelle marker revealed that the internalized Hsp90-SL8C complex localized in Rho B-positive early endosomes (Fig. 7*A*) but not late endosomes/lysosomes or endoplasmic reticulum (ER) after 10, 30, and 60 min of endocytosis (shown for 60 min in Fig. 7*A*). We traced Hsp90-SL8C complex after 90 and 120 min. It accumulated only in early endosomes and did not reach the stage of late endosomes/lysosomes (data not shown). In accordance with experiments using TAP $1^{-/-}$ BMDCs, the lack of accumulation in the ER seemed to

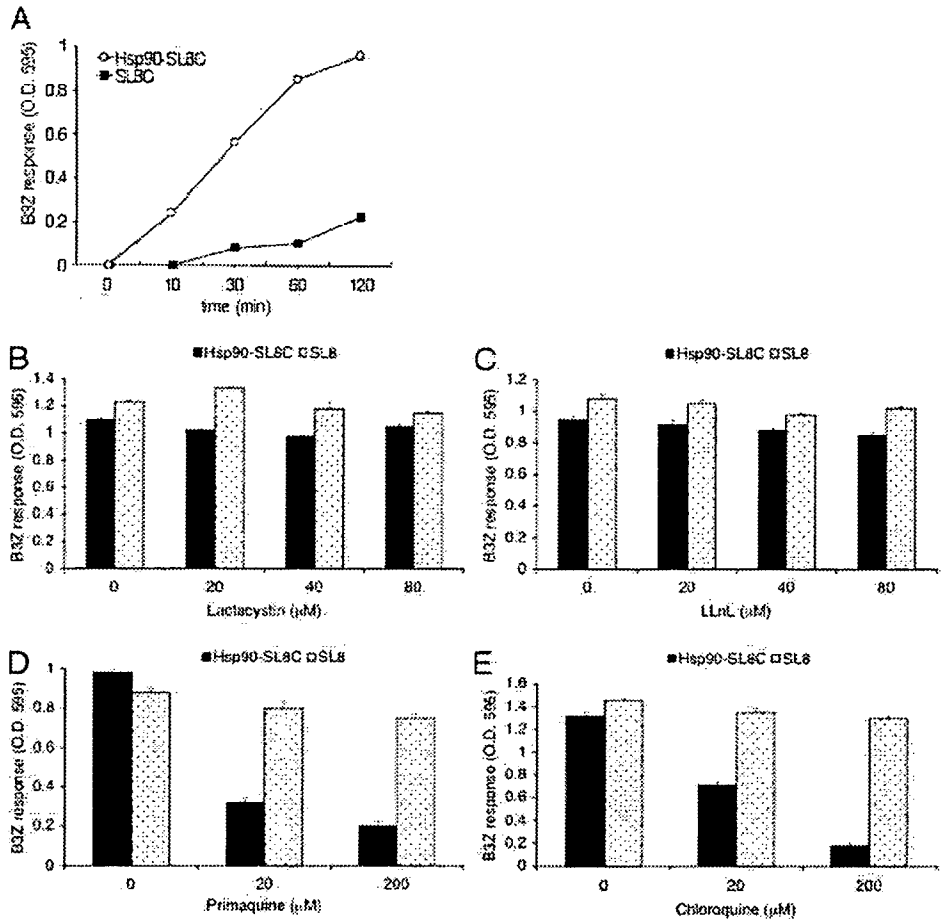

FIGURE 9. Membrane recycling and vacuolar acidification, but not proteasomal processing is required for cross-presentation of Hsp90-peptide complexes. *A*, Cross-presentation of Hsp90-chaperoned peptides by BMDC is very rapid response. *B–D*, BMDCs were preincubated with (*B*) lactacystin, (*C*) LLnL, (*D*) primaquine, or (*E*) chloroquine at 37°C for 2 h, then pulsed with Hsp90-SL8C complexes, SL8 peptide, or OVA protein for 2 h. The DCs were then fixed, washed, and cultured overnight with B3Z cells. The β-galactosidase activity was measured at the absorbance at 595 nm.

indicate that the Hsp90-mediated MHC class I pathway was independent of TAP. We also examined whether Hsp90-peptide complex accumulation in the early endosome was temperature-dependent endocytosis. As expected, at 4°C, labeled Hsp90-peptide complex remained on the cell surface (Fig. 7*B*), but internalization was evident after incubation at 37°C following a 10-min internalization period.

Extracellular Hsp90-peptide complexes and recycling MHC class I molecules are colocalized within early endosomes in BMDCs

To investigate in which compartment the Hsp90-chaperoned antigenic peptides bound to MHC class I molecules, we stained H-2K^b molecules and exogenous Hsp90-SL8C complexes. After 20 min of endocytosis, Alexa Fluor 488-labeled Hsp90-SL8C complexes colocalized with endocytosed H-2K^b molecules in the early endosome (Fig. 8*A*). This finding suggested that Hsp90-bound peptides might be transferred to MHC class I molecules in the early endosome where recycled MHC class I molecules from the plasma membrane are available. The peptide-MHC class I complexes generated in the early endosome would then be transported to the cell surface of the BMDCs, where specific CTLs recognize them.

Early endosomes are the compartment where Hsp90-bound precursor peptides are processed and transferred onto subcellular MHC class I molecules

To investigate in which compartment Hsp90-bound precursor peptides are processed and subsequently transferred onto MHC class I molecules, we used mAb 25D1.11 because this mAb recognizes SL8 peptide-H-2K^b complexes (31). Hsp90-SL8C peptide complexes were pulsed onto BMDCs, subsequently fixed with acetone, and stained with mAb 25D1.11 labeled with Alexa Fluor 488 and anti-Rho B or anti-KEDL Abs coupled with Alexa Fluor 594. Consequently, we clearly observed that mAb 25D1.11 was detected only in the early endosomes and not in the ER (Fig. 8*B*). This fact indicated that Hsp90-bound precursor peptides were processed and transferred onto MHC class I within early endosomes, suggesting that recycling MHC class I molecules are required for efficient presentation of Hsp90-chaperoned peptides.

Kinetics of cross-presentation of Hsp90-chaperoned peptide by BMDCs

We evaluated the cross-presentation kinetics of the Hsp90-chaperoned precursor peptide by BMDCs. BMDCs were pulsed with the Hsp90-SL8C complex, sampled between 0 and 120 min and fixed with glutaraldehyde to terminate further Ag uptake and processing. After fixation, BMDCs were cocultured with B3Z T cell hybridoma. The presentation of Hsp90-chaperoned peptides was detected after a 10-min pulse (Fig. 9*A*), indicating that induction of immune responses can be achieved very rapidly. This is very important for Hsp90 as a danger signal. In contrast, presentation of free SL8C peptide was barely detectable within 2 h. In addition, to confirm that the Hsp90-mediated cross-presentation followed a TAP-independent pathway, we tested the effect of treating BMDCs with the proteasome inhibitor, lactacystin and LLnL. As expected, these agents did not affect the Hsp90-mediated cross-presentation by BMDCs (Fig. 9, *B* and *C*). Therefore, class I-presented peptides