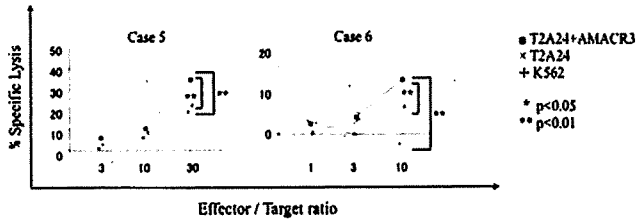

Figure 6
AMACR3 peptide-specific CTL induction from PBMCs of HLA-A24-positive prostate cancer patients. PBMCs of HLA-A24-positive prostate cancer patients (cases 5 and 6) were stimulated four times with three kinds of AMACR peptide (AMACR1-3)-pulsed APCs and their cytotoxic activities were examined by ^{51}Cr release assay at the indicated effector/target ratios. AMACR3 peptide-pulsed T2-A*2402 cells served as target cells. Non-pulsed T2-A*2402 cells and K562 cells were used as negative control target cells.

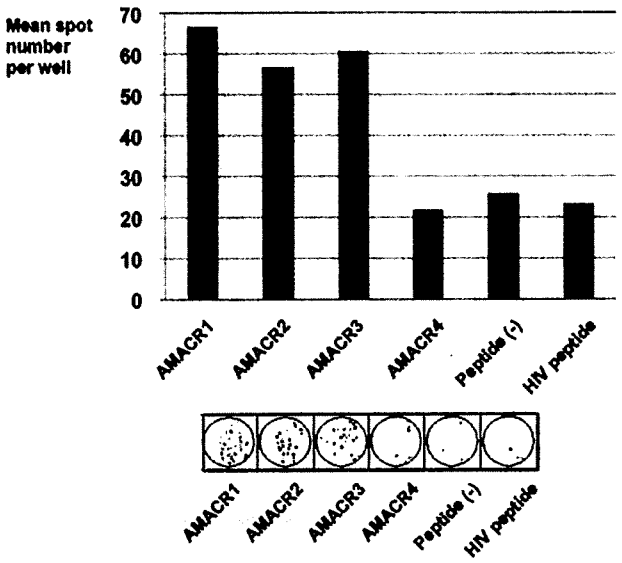

Figure 7
Peptide-specific interferon-Brelease of CTLs. PBMCs of HLA-A24-positive prostate cancer patient (case 6) were stimulated four times with four kinds of AMACR peptide (AMACR1-4)-pulsed APCs and peptide-specific interferon-B release was analyzed by ELISPOT assay. CTLs could release interferon-Bin response to AMACR1, 2 and 3 peptides, but not in response to AMACR4 peptide or HIV peptide.

Because of the cancer specificity and high frequency of AMACR expression, it can be an attractive target for cancer immunotherapy. In this study, the immunogenic potency of AMACR-derived peptides was assessed using PBMCs from prostate cancer patients.

We focused on AMACR-derived peptides carrying the HLA-A24 binding motif. The HLA-A*2402 genotype is predominant in Japanese, accounting for about 60% of the population [28]. Four AMACR-derived peptides (AMACR1-4) carrying the HLA-A24-binding motif were identified in the present study. By stimulating peripheral blood lymphocytes of HLA-A24-positive/AMACR-expressing prostate cancer patients with these AMACR-derived peptides *in vitro*, peptide-specific CTLs were successfully induced in 4 of 9 patients. Moreover, the CTLs exerted significant cytotoxic activity against AMACR-expressing prostate cancer cells in the context of HLA-A24, indicating that AMACR-derived peptides might be useful as prostate cancer vaccines for HLA-A24-positive/AMACR-expressing prostate cancer patients. We demonstrated HLA-A24-restricted CTL responses against AMACR-derived peptides for the first time. Interestingly, the immunogenic peptides were distinct among the patients. However, it is likely that the AMACR2 peptide was the most immunogenic of the three AMACR-derived peptides.

There may be some problems in introducing new CTL-based immunotherapy for advanced recurrent and/or metastatic prostate cancer patients. Even after four rounds of *in vitro* stimulation of PBMCs with the peptides, cytotoxicity against AMACR-expressing tumor cells (% lysis) was only around 20% at a 30:1 E:T ratio. Such weak cytotoxicity may be insufficient to induce a clinical anti-tumor response. Since AMACR is involved in the bile acid synthesis and there is weak expression in the liver, it is possible that T-cells with strong reactivity to AMACR might have tolerance to the antigenic stimulation. Thus, further studies are required to increase the cytotoxic potential of the AMACR-specific CTLs. Moreover, it is reported that AMACR expression is decreased in castration-resistant metastatic diseases [29,30]. In addition, HLA class I expression is decreased in almost 80% of prostate cancer cases as reported by us and other groups [31-33]. The down-regulation of HLA class I was observed more frequently in metastatic sites than in the primary sites. Since HLA class I has a critical role in the recognition of tumor cells by CTLs, defects in antigen presentation could allow the tumor cells to escape from killing by CTLs [34-36]. We showed previously that HLA class I down-regulation was caused at least in part by transcriptional silencing of the β2-microglobulin gene by histone deacetylation in prostate cancer cells, and HLA class I was restored by treatment with histone deacetylase inhibitors [33]. It may be possible for CTL-based vaccines to be used in combination with histone deacetylase inhibitors in immunotherapy for prostate cancer.

Conclusion

In conclusion, we have provided evidence that AMACR is a potent immunogenic antigen for prostate cancer and

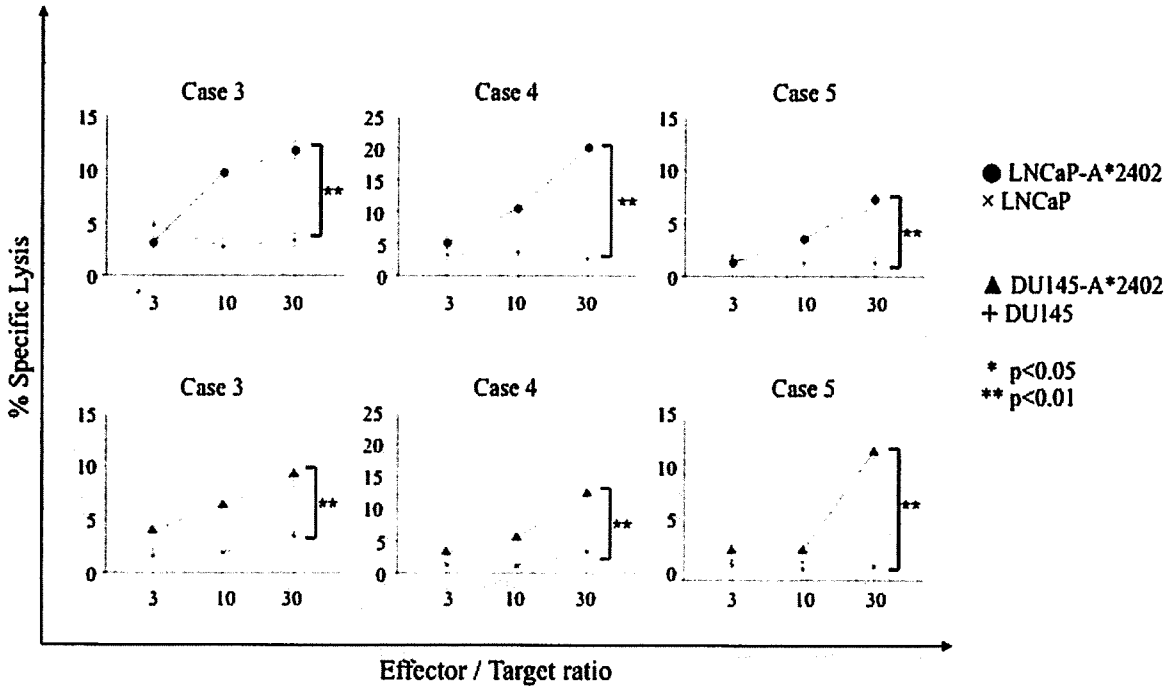

Figure 8
Cytotoxic activities of AMACR peptide-specific CTLs against HLA-A24-positive AMACR-expressing prostate cancer cell lines. AMACR peptide-specific CTLs were examined for the cytotoxic activity against HLA-A24-positive AMACR-expressing prostate cancer lines, LNCaP-A*2402 and DU145-A*2402, which were stable HLA-A*2402-transfectants of LNCaP and DU145, respectively. The cytotoxicity was assessed by ^{51}Cr release assay at the indicated effector/target ratios.

AMACR-derived peptides might serve as a cancer vaccine for HLA-A24-positive prostate cancer patients. It is possible that AMACR-targeting therapy might become a rational modality in immunotherapy for various AMACR-expressing cancers.

Abbreviations

AMACR: alpha-methylacyl-CoA racemase; CTL: cytotoxic T-lymphocyte; PBMC: peripheral blood mononuclear cells; DC: dendritic cell; PHA: phytohemagglutinin; APC: antigen presenting cell.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IH carried out the CTL induction, killing assays and drafted the manuscript. TT and YH participated in the design of the study and performed the evaluation of the data. TT helped to draft the manuscript. YH contributed to the HLA-A24-binding assay and CTL induction from PBMCs. HK, ES and NM contributed to collecting patients' samples with the informed consent. YT, TT and NS contributed to the design and coordination of this study as well as reviewing the manuscript. All authors have read and approved the final manuscript.

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RESEARCH ARTICLE

Heat shock proteins and immunity: Application of hyperthermia for immunomodulation

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Abstract
Heat shock proteins (HSPs) play an important role as 'endogenous danger signals' in the immune surveillance system. Extracellular HSPs released from damaged cells can stimulate professional antigen-presenting cells, followed by cytokine release and expression of cell surface molecules. In addition to such activity stimulating innate immunity, extracellular HSPs can promote the cross-presentation of HSP-bound peptide antigens to MHC class I molecules in dendritic cells, leading to efficient induction of antigen-specific cytotoxic T-lymphocytes. The roles of HSPs stimulating both innate immunity and adaptive immunity can explain at least in part the molecular mechanism by which thermal stress bolsters the host immune system. In the present review, we present novel aspects of the roles of HSPs in immunity and discuss the therapeutic application of hyperthermia for immunomodulation.

Keywords: *heat shock and immune response, immunotherapy, MHC class I, dendritic cells, antigen presentation*

The role of intracellular HSPs in antigen processing and presentation

Heat shock proteins (HSPs) act as molecular chaperones inside cells, regulating conformational change, translocation, assembly and degradation of cellular proteins. They have important roles in cellular protection against various stresses such as ischaemia, heat stress and oxidative stress [1–3]. They are also involved in the antigen processing and presentation machinery as chaperones for antigenic proteins and peptides. A number of studies have shown that the 70 kDa HSP family (Hsp70) and 90 kDa HSP family (Hsp90) are associated with antigenic peptides in the cytosol and mediate their translocation and processing [4]. We have demonstrated previously that Hsp70 is associated with transporters associated with antigen processing (TAP) and mediates ATP-dependent transportation of antigenic peptides from cytosol to endoplasmic reticulum (ER) [5]. The efficiency of the transportation is correlated with affinity of the peptides to Hsp70, indicating that HSPs might serve as intracellular antigen transporters. HSPs are also associated with proteasomes, which degrade cellular proteins and produce antigenic peptides [6] (Figure 1). In virus-infected cells, viral proteins bind to HSPs to utilise the protein folding machinery. However, some HSP-bound viral proteins are degraded by proteasomes and presented to MHC class I, leading to recognition of the infected cells by cytotoxic T-lymphocytes (CTL). Therefore, increased body temperature and the subsequent HSP induction are important reactions in the host defence system.

The role of extracellular HSPs in innate immune responses

Pattern recognition molecules have crucial roles in innate immune responses. So far, a number of Toll-like receptor (TLR) ligands have been reported, including lipopolysaccharides, peptidoglycans, CpG oligodeoxynucleotides and double-stranded RNA [7].

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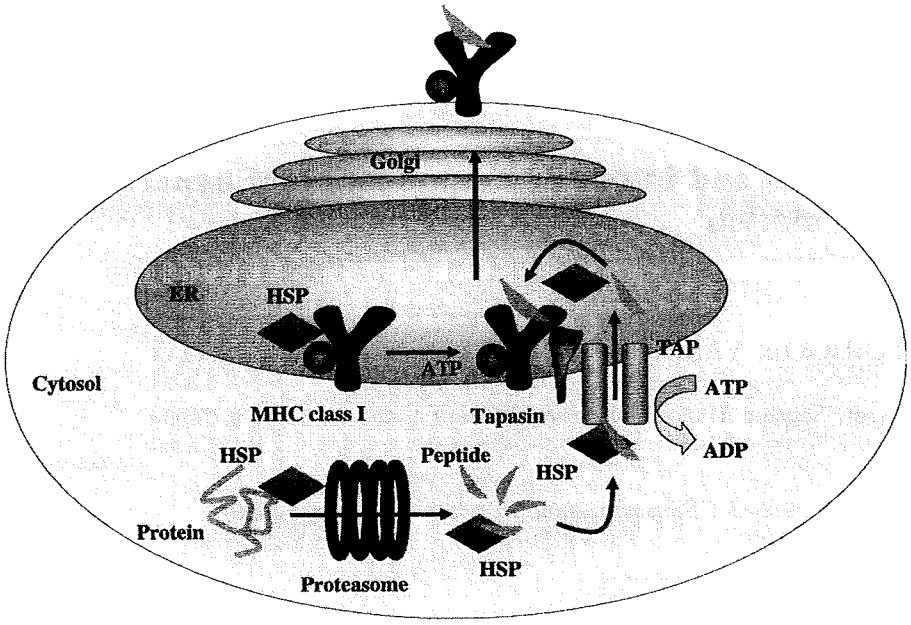

Figure 1. The role of intracellular HSPs in MHC class I antigen presentation. Cellular proteins are degraded by proteasomes, resulting in production of antigenic peptides. The peptides are transported from the cytosol into the endoplasmic reticulum (ER) by transporters associated with antigen processing (TAP), followed by binding to MHC class I molecules and presentation on the cell surface. Molecular chaperone HSPs are associated with antigenic peptides, proteasomes, TAP and MHC class I in this pathway.

In addition to the TLR ligands derived from microorganisms, silica crystals, aluminum salt and asbestos are known to stimulate NOD family molecules [8–11]. It is well known that most of these exogenous foreign molecules activate dendritic cells (DCs) and induce release of cytokines, including TNF-α, interferon and IL-12. Recently, it was revealed that extracellular HSPs could activate DCs as well as exogenous TLR ligands [12, 13]. Hsp70 and Hsp60 are reported to stimulate DCs through TLR4. We have reported that stimulation of DCs with purified Hsp70 results in induction of TNF-α release in a dose-dependent manner, which is inhibited in the presence of Hsp70-targeting polyamine compound deoxyspergualin [14]. Though it has been argued that the pro-inflammatory effects of extracellular HSPs might be mediated through contamination of LPS or other microbial compounds [15], it is true that some aspects of TLR activation by HSPs differ from TLR activation by microbial compounds. For example, activation of TLR signalling by Hsp60 requires endocytosis, whereas that by LPS does not [16]. In another study, it was shown that Hsp70 triggered calcium-mediated signalling in DCs, whereas it was not observed in LPS-triggering signals through TLR [17]. In order to ensure that LPS contamination with Hsp70 was not responsible for the release of cytokines, Hsp70 was either boiled at 95 °C or treated with proteinase K; however, both of these treatments abrogated Hsp70-induced, but not LPS-induced, release of cytokines, providing further evidence against endotoxin contamination contributing to DC activation [17]. Therefore, HSPs are now recognised as 'endogenous danger signals' that can alert the innate immune system in response to cellular damage. The molecular chaperone Hsp70, with cytokine-like activity in relation to DCs, was termed a 'chaperokine' [18]. HSPs released from damaged cells can activate DCs at the site of the injury and induce inflammatory cytokine release (Figure 2). Since the magnitude of the innate immune response is correlated with the amount of extracellular HSPs, more damaged cells and more severe stress can elicit more robust immune responses [14]. Therefore, local hyperthermia treatment might enhance the innate immune response through induction of a heat shock response and extracellular release of HSPs [13].

The role of extracellular HSPs in adaptive immunity

It has been shown that Hsp70 and Gp96, a member of the Hsp90 family, extracted from tumour cells can elicit anti-tumour CTL responses after vaccination in mouse models [19]. The immunogenic tumour antigens recognised by the CTLs are not HSPs themselves, but HSP-bound proteins and peptides [20]. Since HSPs are associated with

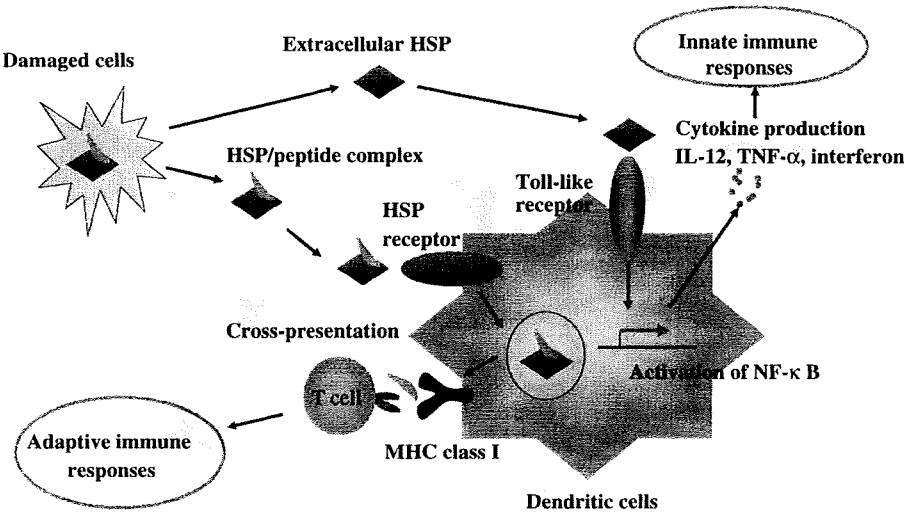

Figure 2. The role of extracellular HSPs in immune responses. Extracellular HSPs can stimulate Toll-like receptors, leading to activation of dendritic cells and release of cytokines such as IL-12, TNF-α and interferon (innate immune responses). Extracellular HSPs can be internalised through HSP receptors with HSP-bound peptides. The antigenic peptides are then cross-presented to MHC class I molecules, leading to induction of peptide-specific CTL responses (adaptive immune responses).

many cellular proteins, especially denatured proteins or mutated proteins in tumour cells, the antigenic proteins can be taken into professional APC in association with HSPs and presented to MHC class I molecules, leading to antigen-specific CTL induction [21, 22]. MHC class I presents peptides derived from endogenous proteins in non-APC. On the other hand, MHC class I can present peptides derived from exogenous proteins in professional APC as well, which is termed 'cross-presentation'. Extracellular HSPs were suggested to enhance cross-presentation of HSP-bound antigens to MHC class I in DCs. However, it remained unclear for a long time how HSPs could facilitate the cross-presentation and induction of CTLs. In 2000 it was reported that a Gp96-peptide complex could be taken into DCs via receptor-mediated endocytosis, and the receptor for Gp96 was CD91, an alpha2-macroglobulin receptor [23]. Basu et al. showed that not only Gp96, but also Hsp90, Hsp70 and calreticulin used CD91 as a common receptor [24]. Following the initial reports, other molecules were shown to be receptors for Hsp70 and Gp96, including CD40 [25] and the scavenger receptor family members LOX-1 [26] and SR-A [27]. Thus, it became evident that DCs could internalise HSP-chaperoned proteins and peptides through various receptors by endocytosis [28, 29]. We have analysed the antigen-processing pathway for cross-presentation after endocytosis. We showed that internalised Hsp70 or Hsp90 was transported preferentially into the early endosome and not to the ER or lysosome in DCs [30]. HSP-bound antigens are then processed in the endosome, followed by presentation through recycling MHC class I molecules (endosomal pathway), or translocated into the cytosol, followed by processing through proteasome-TAP machinery and presentation through MHC class I in the ER (TAP-ER pathway) [31–38] (Figure 3). It is proposed that DCs may have a unique membrane-transport pathway linking the endosomal compartment to the cytosolic compartment [39].

In addition, we have found that HSPs, especially Hsp90, have a potent endosome-targeting capability in professional APCs [38, 40]. Hsp90-chaperoned proteins were presented much more selectively through the MHC class I pathway (early endosomal pathway) than through the MHC class II pathway (late endosomal pathway). In contrast, free proteins are presented preferentially through the MHC class II pathway but not through the MHC class I pathway, resulting in antibody responses rather than CTL responses.

These studies clarified novel roles of extracellular HSPs in adaptive immunity. Professional APCs can present extracellular HSP-bound peptides/proteins to MHC class I and induce antigen-specific CTL responses. The HSP-mediated cross-presentation is more rapid and efficient than free antigens, indicating that extracellular HSPs can activate not only innate immune responses but also adaptive immune responses.

Application of HSPs for vaccine development

On the basis of the immunostimulatory activity of HSPs, we examined the application of HSPs for

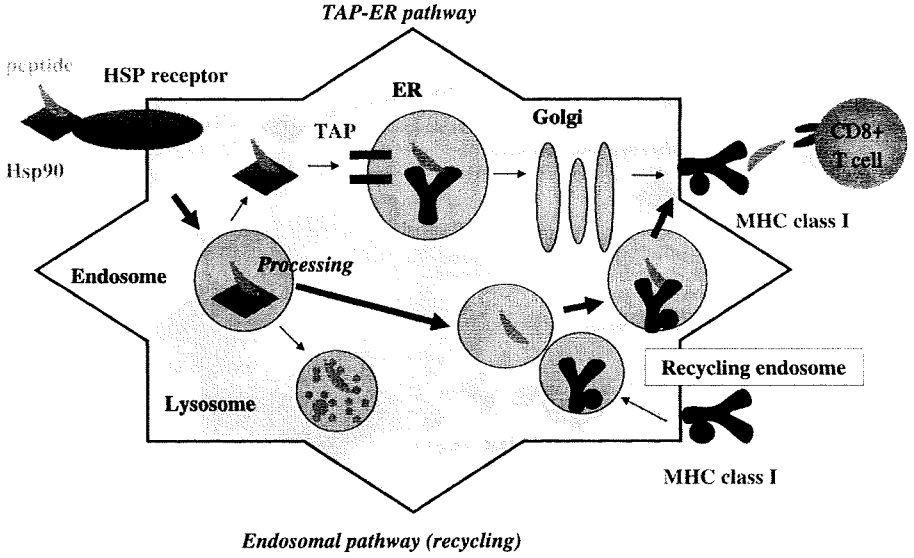

Figure 3. Cross-presentation pathway of Hsp90-antigen complex. HSP-antigen complexes internalised through HSP receptors are transported into the early endosome, followed by processing and presentation through MHC class I in the recycling endosome. Some of the HSP-antigen complexes may be released into the cytosol, followed by transportation through TAP and presentation through MHC class I in the ER.

vaccine adjuvant. Activation of DCs is required to achieve an efficient immune response to vaccination. Thus far, some TLR ligands such as CpG oligodeoxynucleotides and peptidoglycans have been employed as adjuvants as well as classical Freund adjuvants. However, most of the trials failed due to severe adverse effects or lack of effectiveness. The safe and common adjuvants in clinical use at present are mineral oil (Freund incomplete adjuvant) and aluminium. In animal models we compared the efficiency of peptide-specific CTL induction among peptide vaccines with various compositions such as peptide + PBS, peptide + Freund adjuvant, peptide + CpG oligodeoxynucleotide, peptide + Hsp70 and peptide + Hsp90 [38]. It was demonstrated that efficient CTL induction was achieved via vaccination with peptide + Hsp90 or peptide + complete Freund adjuvant (CFA) (Figure 4). However, vaccination with peptide + CFA caused severe local inflammation with skin ulceration. In contrast, there was no obvious side effect in the case of Hsp90 vaccination, indicating the superior safety and immunostimulatory action of Hsp90. Successful immunisation was also demonstrated in a mouse tumour therapeutic model. Vaccination of tumour-bearing mice with peptide + Hsp90 resulted in tumour regression and increased survival [38]. The results represent the greater advantage of utilising 'endogenous danger signals' in the development of vaccine as compared to 'exogenous danger signals', which are less physiological. Our study provides a rationale for a novel vaccine strategy in the field of cancer and infective diseases.

Application of hyperthermia for immunomodulation

It has been reported that hyperthermia in the febrile range could induce heat shock responses and subsequent HSP expression in human cells [41]. Therefore, the roles of intracellular and extracellular HSPs in the immune system can explain at least in part the benefits of fever in infectious diseases [42]. In addition to the HSP-family genes, expression of a number of immunomodulatory genes are induced during febrile-range hyperthermia, including cell adhesion molecules such as ICAM-1/CD54, JAM3, CD11b and CD47, TLRs such as TLR-6 and TLR-7, chemokines such as CXCL-5, CXCL-7 and IL-8, and prostaglandin E synthase [43]. There is accumulating evidence that fever-range thermal stress bolsters primary immune surveillance of lymphoid organs by augmenting lymphocyte extravasation across specialised blood vessels termed high endothelial venules (HEVs) [44–47]. Chen et al. showed that thermal stress enhanced endothelial expression of ICAM-1/CD54 and CCL21 chemokine, leading to increased lymphocyte trafficking across HEVs [48–50]. These mechanisms substantially increase the probability of antigen-specific T cells encountering the APCs in lymphoid organs. They also revealed that one of the important mediators of thermal effects upon lymphocytes and HEVs was IL-6 trans-signalling [48, 49]. These data suggest that hyperthermia treatment that is clinically applied as adjuvant treatment for sarcoma, melanoma and cervical cancer might be effective in the

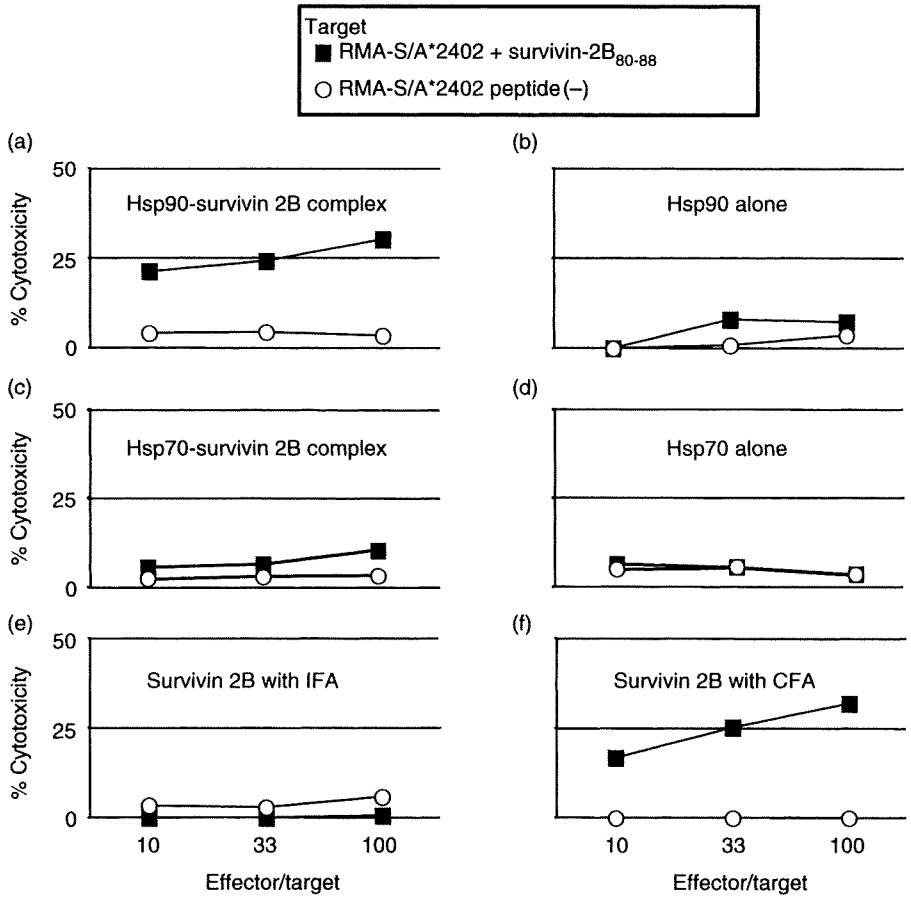

Figure 4. Hsp90-peptide vaccine can induce an efficient specific CTL response. HLA-A*2402/Kb-transgenic mice were immunised four times with the indicated peptide vaccination. Spleen cells were removed one week after the last immunisation, cultured for five days with survivin-2B80–88 peptides, and tested 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-2B80–88 peptide, or without the peptide. Note that CTL were induced in the cases of vaccination with Hsp90 + peptide and complete Freund adjuvant (CFA) + peptide. IFA: incomplete Freund adjuvant. (Figure from reference [38]). Copyright 2007. The American Association of Immunologists, Inc.

enhancement of anti-tumour immune responses [51–53]. CTL-inducible HSP vaccine immunotherapy might be developed in combination with hyperthermia. However, the optimum temperature and duration of hyperthermia for the purpose of immunomodulation remain unclear and have to be determined through further studies, since they are quite distinct from the cytotoxic conditions utilised in the field of cancer therapy. Thermal effects on the immune responses can be achieved in the fever-range temperature (38–41 °C) [48], whereas cytotoxic effects of thermal stress can be achieved in the non-physiological range temperature (over 42 °C). In addition, it has been reported that thermal stress in certain condition can suppress the innate immune responses in macrophages [54, 55].

It is expected that hyperthermia and HSP vaccine might be applicable to the treatment of autoimmune diseases such as type I diabetes and rheumatoid arthritis [56]. The rationale came from the evidence that self-HSP-specific CD4-positive T-cells have been found in association with chronic inflammatory diseases and the HSP-specific T-cells have an immunoregulatory phenotype that can suppress immune responses in autoimmune diseases [56–58]. Indeed, there have been some clinical trials of HSP vaccine for the treatment of rheumatoid arthritis and type I diabetes [59, 60]. There is a report of an animal model showing that whole-body hyperthermia could attenuate autoimmune myocarditis [61]. It is suggested that hyperthermia-mediated induction of endogenous HSPs might facilitate the induction of CD4-positive immunoregulatory T-cells. As we discussed above, Hsp90-bound antigenic peptides could elicit strong peptide-specific CD8-positive cytotoxic T-cell responses through the stimulation of DCs. However, Hsp60 and Hsp70 could induce CD4-positive regulatory T-cell responses in certain conditions [56]. It seems that the direction of HSP-mediated immune response differs among different HSP family proteins and different HSP-bound antigens. Therefore, it is possible that hyperthermia

treatment causes a distinct effect on the immune response, either immunogenic or tolerogenic, depending on the tissue and the temperature. Though further studies will be required to develop a novel therapeutic strategy, there are promising advances in the fields of thermal medicine and immunotherapy.

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Targeting to Static Endosome Is Required for Efficient Cross-Presentation of Endoplasmic Reticulum-Resident Oxygen-Regulated Protein 150-Peptide Complexes[1]

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Heat shock proteins (HSPs) such as Hsp70, gp96, and Hsp90 have been shown to elicit intriguing, efficient CTL responses by cross-presentation via an as yet entirely unknown mechanism. Oxygen-regulated protein 150 (ORP150), also known as grp170, is an endoplasmic reticulum-resident HSP and is up-regulated by hypoxia. It has been demonstrated that ORP150 binds tumor-associated Ag peptides within cancer cells. Immunization with an ORP150-tumor Ag complex has been shown to generate tumor-specific CTLs. Most recently, it has been shown that exogenous ORP150 induces cross-presentation of a chaperoned Ag, thereby stimulating Ag-specific CTLs. However, the mechanism underlying this efficient cross-presentation is still unsolved. In this study, we show that the ORP150-precursor peptide complex can elicit CTL response through cross-presentation as well as the $CD4^+$ T cell response by dendritic cells. Furthermore, we observed that the internalized ORP150-peptide complex, but not OVA protein, which was not cross-presented, was sorted to the $Rab5^+$, $EEA1^+$ static early endosome, followed by translocation to a recycling endosome, where the ORP150-chaperoned peptide was processed and bound to MHC class I molecules. Moreover, we observed that immunization of mice with ORP150-peptide complexes elicited strong peptide-specific CTLs and antitumor effects in vivo. Our data indicate that targeting of the Ag to a "static" early endosme by ORP150 is required for the efficient cross-presentation. *The Journal of Immunology,* **2009, 183: 5861–5869.**

It is well known that tumor-derived heat shock proteins (HSPs)[3] such as Hsp70, Hsp90, and gp96 initiate efficient tumor-specific CTL responses and protective immunity (1–5). Although immunized HSPs are exogenous Ags, these HSP-Ag complexes can gain access to the class I Ag presentation pathway, resulting in the stimulation of $CD8^+$ T cells, termed cross-presentation (6–11). The ability of HSPs to facilitate the cross-presentation of MHC class I-restricted epitopes and to prime $CD8^+$ T cell responses is due to the following: (1) HSPs are able to form stable complexes with antigenic peptides/proteins, (2) HSP-peptide/protein complexes are able to bind surface receptors on APCs, resulting in receptor-dependent endocytosis, and (3) HSP can stimulate an innate immune response, which is not dependent on tumor Ags (12, 13). It is thought that HSPs bind to receptors on APCs, resulting in secretion of proinflammatory cytokines and maturation and activation of dendritic cells (DCs). To date, CD91 (14–16), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) (17), CD40 (18), scavenger receptor type A (SR-A) (19, 20), and scavenger receptor expressed by endothelial cells-I (SREC-I) (20, 21) have been demonstrated to be receptors for several kinds of HSPs expressed on APCs.

DCs are the most potent APCs for efficient cross-presentation. Recently, it has been shown that DCs can internalize HSP-peptide complexes by receptor-mediated endocytosis and direct chaperoned 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 (10). However, the underlying mechanism for efficient cross-presentation, in particular how the HSP-Ag complex can enter the MHC class I pathway, is not well understood. Recently, we have demonstrated that extracellular Hsp90-peptide complexes are efficiently cross-presented via the endosome-recycling pathway (22). In this Hsp90-mediated cross-presentation, the receptor-dependent endocytosed Hsp90-peptide complex was transferred to the early endosome in which a cysteine protease such as cathepsin S processed the precursor peptide. The resulting MHC class I epitope was transferred onto recycling MHC class I molecules, thereby expressing an MHC class I-epitope complex on the cell surface. Furthermore, we have shown that immunization with Hsp90-tumor Ag peptide complexes induces Ag-specific CTL responses and strong antitumor immunity in vivo.

Oxygen-regulated protein 150 (ORP150), also known as glucose-regulated protein 170 (grp170), was first described in 1996 by Kuwabara et al. (23). It is an endoplasmic reticulum (ER)-resident Hsp70 superfamily member, and it is induced by stress conditions such as hypoxia, ischemia, glucose deprivation, reductive reagents,

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[3] Abbreviations used in this paper: HSP, heat shock protein; BMDC, bone marrow-derived dendritic cell; CPRG, chlorophenol red-β-D-galactopyranoside; DC, dendritic cell; ER, endoplasmic reticulum; HPF, high-power field; LAMP-1, lysosome-associated membrane protein 1; ORP150, oxygen-regulated protein 150; SR-A, scavenger receptor type A; SREC-I, scavenger receptor expressed by endothelial cells-I.

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and interference with calcium homeostasis (24). Subjeck's group has performed extensive research and shown that the ORP150 induces antitumor immunity in vivo (25–28). Vaccination with ORP150 purified from tumors suppresses the same tumor growth in mice and induces tumor-specific CTLs. Furthermore, it has been demonstrated that ORP150 induces efficient cross-presentation of chaperoned Ags, thereby stimulating the Ag-specific CTLs (28). Moreover, the receptor for ORP150 has been shown to be SR-A and SREC-I (20). However, the precise intracellular mechanism for cross-presentation of ORP150-peptide complexes is not well understood.

In this study, we first examined whether an ORP150-precursor peptide complex could elicit a strong peptide-specific CTL response and antitumor immunity through cross-presentation using bone marrow-derived DCs (BMDCs) as APCs. Furthermore, we analyzed the intracellular trafficking pathway of the ORP150-peptide complex for efficient cross-presentation within DCs. Recently, Lakadamyali et al. have shown that early endosomes are comprised of two distinct populations, called "static" early endosome, which is slow maturing, and rapidly maturing "dynamic" early endosome (29). Furthermore, Burgdorf et al. have demonstrated that the mannose receptor introduced OVA specifically into an $EEA1^+$, $Rab5^+$-static early endosomal compartment for subsequent cross-presentation. In contrast, pinocytosis conveyed OVA to lysosome for class II presentation (30). Our observations revealed that targeting of the ORP150-peptide complex to the $EEA1^+$, $Rab5^+$-static early endosome is crucial for cross-presentation. We propose that the static early endosome plays an important role in HSP-mediated cross-presentation by DCs.

Materials and Methods

Mice

Female B6C3F1 ($H\text{-}2^{b/k}$) mice, C57BL/6 ($H\text{-}2^b$) mice, and $TAP1^{-/-}$ ($H\text{-}2^b$) mice were purchased from The Jackson Laboratory and used at 6 wk of age. Mice were maintained in a specific pathogen-free mouse facility at Sapporo Medical University according to institutional guidelines for animal use and care.

Cells

The B3Z cell is a $CD8^+$ T cell hybridoma specific for the $OVA_{258-265}$ epitope (SL8) in the context of $H\text{-}2K^b$. The KZO cell is a $CD4^+$ T cell hybridoma specific for the $OVA_{247-265}$ (PL19) in the context of $I\text{-}A^k$. These hybridomas synthesize a reporter enzyme when the TCR engages the $H\text{-}2K^b$-SL8 (SIINFEKL) complex or the $I\text{-}A^k$-PL19 (PDEVSGLEQLESI INFEKL) complex, respectively. The cell lines were obtained from Dr. N. Shastri (University of California at Berkeley, 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). TG3 cells are a methylcholanthrene-induced fibrosarcoma derived from the HLA-A*2402 transgenic mouse. TG3–2B cells were TG-3 transfected with the gene encoding human survivin-2B. YAC-1 cells and 293T cells were obtained from the American Type Culture Collection.

Generation of BMDCs

Bone marrow-derived immature DCs were generated from bone marrow cells that were obtaind from the femurs and tibiae of female C57BL/6 mice, B6C3F1 mice, and $TAP^{-/-}$ mice. Bone marrow cells (1×10^4/well) in a 24-well plate were cultured in complete RPMI 1640 with 10% FCS and 20 ng/ml GM-CSF (Endogen) for 5 days. Medium with GM-CSF was gently replaced on day 2 and day 4.

Construction of secreted form of ORP150 and generation of 293T cells secreting ORP150

Using human ORP150 cDNA as a template, the sense primer (5′CGG GATCCATGGCAGACAAAGTTAGGAGG-3′) and the antisense primer (5′-GGACAGAAGCGGCCTTTGAAGGCGGCCGCCG-3′) were used to generate an $NDEL^-$ ORP150 cDNA. The PCR product was digested with *Bam*HI/*Not*I and ligated into pIRESpuro3-myc/His vector (Clontech Laboratories). The sequence of the construct was verified by DNA sequencing. 293T cells were transfected with this construct using Lipofectamine 2000 (Invitrogen). Cells were then subcloned with 1 mg/ml puromycin (Invitrogen) and a 293T cell-secreting ORP150, 293T-ORP4, was established.

Purification of secreted ORP150

293T-ORP4 cells were cultured with 10% DMEM for 3 days. Then, the culture supernatant of 293T-ORP4 cells was collected and applied to a Con A-agarose column (Amersham Biosciences). After extensive washing of the column with Con A-PBS (150 mM NaCl, 5 mM NaP, 1.35 mM KCl, 1.35 mM $MgCl_2$), ORP150 was eluted off with Con A-PBS containing 10 mM methyl α-D-mannopyranoside (Sigma-Aldrich). The presence of ORP150 in the eluted fraction was examined by SDS-PAGE stained with Coomassie brilliant blue (Bio-Rad) and Western blotting using a mAb against ORP150 (IBL). Typically, fractions 2–5 contained purified ORP150 and these fractions were pooled and concentrated using Microcon YM-100 (Millipore). The concentration of ORP150 was determined using a Micro BCA protein assay reagent kit (Pierce). Quantification of the endotoxin in the purified ORP150 was performed using the endotoxin-specific chromogenic test (ES test; Seikagaku Kogyo). If the endotoxin in the ORP150 preparation was higher than the limit of detection (<5 pg/ml), endotoxin was depleted using Detoxi-Gel endotoxin removing gel (Pierce) and then requantified.

Peptides and proteins

The following peptides were used (underlined sequences represent the precise MHC class I- or class II-binding epitope): survivin-$2B_{80-88}$ (AYACNTSTL), SL8 (SIINFEKL), SL8C (SIINFEKLTEWTS), PL19 (PDEVSGLEQLESIINFEKL), PLC24 (PDEVSGLEQLESIINFEKLTEWTS), and SLC26 (SIINFEKLTEWTSSNVMEERKIKVYL). All peptides were synthesized on a solid phase support using F-moc for transient NH_2-terminal protection and were characterized using mass spectrometry. They were purified by HPLC to >99% homogeneity and stored at 2 mM in distilled H_2O at −80°C. BSA was purchased from Sigma-Aldrich. Chicken OVA was purchased from Calbiochem and stored at 20 mg/ml in PBS at −80°C.

Antibodies

Confocal laser microscopy was used to detect organelles with specific Abs: an anti-Rab5 pAb (MBL) and EEA1 (Abcam) for early endosomes, anti-lysosome-associated membrane protein 1 (LAMP-1) pAb (Santa Cruz Biotechnology) for late endosomes/lysosomes, anti-Rab11 pAb (Santa Cruz Biotechnology) for recycling endosomes, and anti-KDEL mAb (StressGen Biotechnologies) for ER. mAb 25D-1.16 specific for the $K^b/OVA_{257-264}$ complexes was provided by Dr. R. Germain (National Institutes of Health, Bethesda, MD). The Abs were each conjugated with Alexa Fluor 488 (Molecular Probes) according to the manufacturer's instructions. Alexa Fluor 594 (Molecular Probes) was used for labeling ORP150.

Generation of ORP150-peptide complex in vitro

Peptide SLC26 (SIINFEKLTEWTSSNVMEERKIKVYL) was iodinated with ^{125}I using IODO-BEADS (Pierce) according to the manufacturer's instructions. ORP150 or control protein BSA was mixed with the ^{125}I-labeled peptide in a 50:1 peptide-to-protein molar ratio in 0.7 M NaCl containing sodium-phosphate buffer and heated at 45°C for 30 min, then incubated for 30 min at room temperature. The complex was separated from unbound peptide using a Microcon YM-100 or YM-30 (Millipore). Radioactivity of the complex and unbound peptide was measured in a gamma counter for evaluation of the binding efficiency of the ORP-peptide complex. Samples were then analyzed by SDS-PAGE, followed by autoradiography of the stained gel.

In vitro cross-presentation assay

DCs (1×10^5) from C57BL/6 or $TAP^{-/-}$ mice were pulsed with PLC24 (20 μg) alone, different ORP150/peptide dose ratios, or SL8 (1 μM) for 2 h at 37°C in 100 μl of Opti-MEM, and fixed for 1 min with 0.01% glutaraldehyde. Fixation was stopped by addition of 2 M L-lysine and the cells were washed twice with RPMI 1640 medium and cultured overnight with 1×10^5 B3Z. The B3Z response was measured as β-galactosidase activity induced upon ligand recognition. The β-galactosidase activity was measured by the absorbance at 595 nm of the cleavage product of chlorophenol red-β-D-galactopyranoside (CPRG; purchased from Roche). In other experiments, DCs (1×10^5) from C57BL/6 or $TAP^{-/-}$ mice were pulsed with ORP150 (20 μg) alone, PLC24 (20 μg) alone, a complex of both generated in vitro, a simple mixture of both, OVA (200 μg/ml), or SL8 (1 μM) for 2 h at 37°C in 100 μl of Opti-MEM, fixed, and then cocultured overnight with 1×10^5 B3Z.

Ag presentation assay

DCs (1×10^5) from B6C3F1 mice were pulsed with ORP150 (20 μg) alone, PLC24 (20 μg) alone, a complex of both generated in vitro, a simple mixture of both, OVA (200 μg/ml), or PL19 (1 μM) for 2 h at 37°C in 100 μl of Opti-MEM, and fixed for 1 min with 0.01% glutaraldehyde. Fixation was stopped by addition of 2 M L-lysine and the cells were washed twice with RPMI 1640 medium and cultured overnight with 1×10^5 KZO. The KZO response was measured as β-galactosidase activity using CPRG by the absorbance at 595 nm.

In vivo cross-presentation assay

C57BL/6 mice were immunized in their footpads with ORP150 (50 μg) alone, SL8C (50 μg) alone, or the ORP150 (50 μg)-SL8C (50 μg) complex. Draining popliteal lymph nodes were removed after 12 h, and $CD11c^+$ DCs were purified using CD11c MACS beads (Miltenyi Biotec). Purified DCs were plated at a density of 1×10^3/200 μl in 10% RPMI 1640 and cocultured overnight with 1×10^5 B3Z. Stimulated B3Z cells were stained with CPRG and red color was measured as absorbance at 595 nm.

Inhibition studies

DCs (1×10^5) from B6C3F1 mice were preincubated with primaquine (ICN Biomedicals). Primaquine was not toxic in our culture systems. Two hours after preincubation, the DCs were pulsed with the ORP150 (20 μg)-PLC24 (20 μg) complex, SL8 (1 μM), or PL19 (1 μM) for 2 h at 37°C in 100 μl of Opti-MEM, then fixed, washed, and cultured overnight with B3Z or KZO. Stimulated B3Z or KZO were stained with CPRG and red color was measured as absorbance at 595 nm.

Immunocytological localization of ORP150-SL8C complex

ORP150, transferrin (Molecular Probes), and OVA were conjugated with Alexa Fluor 594 (Molecular Probes) according to the manufacturer's instructions. Immature BMDCs were incubated at 37°C with Alexa Fluor 594-labeled ORP150 (20 μg) complexed with PLC24 (20 μg) for 1 h. Following incubation, cells were washed twice with ice-cold PBS and fixed with ice-cold acetone for 1 min. Organelles were stained with an anti-Rab5 pAb and EEA1 mAb for early endosomes, anti-LAMP-1 pAb for late endosomes and lysosomes, anti-Rab11 pAb for recycling endosomes, or anti-KDEL mAb for ER, followed by Alexa 488-conjugated goat anti-rabbit IgG or anti-mouse IgG and visualized with a Bio-Rad MRC1024ES confocal scanning laser microscope system. For detecting the intracellular localization of H-$2K^b$/SL8 complex using mAb 25D-1.16, the DCs were first incubated with ORP150 complexed with PLC24 for 1 h and fixed with cold acetone. DCs were then incubated with anti-mouse CD16/CD32 Fc-block to block nonspecific staining, followed by costaining with an Alexa Fluor 594-labeled mAb 25D1.16 and anti-organelle Abs conjugated with Alexa Fluor 488. For evaluation of colocalization, single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 90 cells (30 cells from three independent experiments) were analyzed.

^{51}Cr-release assay

Each HLA-A*2402/K^b transgenic mouse was immunized s.c. at the base of the tail, twice with a 1-wk interval, with the ORP150 (50 μg)-survivin-2B peptide (50 μg) complex. One week after the last immunization, splenocytes of immunized mice were cultured with irradiated (100 Gy) and survivin-$2B_{80-88}$ peptide-pulsed naive 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 was evaluated using TG3–2B cells, RMA-S-A*2402 cells, RMA-S-A*2402 cells pulsed with 1 μg/ml survivin-$2B_{80-88}$ peptide, and YAC-1 cells as targets.

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 3–4 mm, the mice were then treated with ORP150 (50 μg) alone, the ORP150 (50 μg)-survivin-$2B_{80-88}$ peptide (50 μg) complex, or survivin-$2B_{80-88}$ peptide (50 μg) via s.c. administration at the nape of the neck twice each week for 2 wk (on days 9, 12, 17, and 21). 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. On day 35, tumor rejection rates were compared among the groups. Average tumor diameters on day 28 were statistically analyzed using the Mann-Whitney *U* test. Statistical analyses for evaluating the survival advantages were performed using log-rank analysis. All of the experiments were performed with 8–10 mice per group.

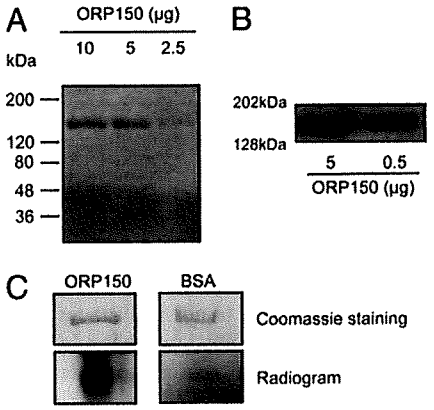

FIGURE 1. Purification of secreted form of ORP150. *A*, Purified ORP150 (10, 5, or 2.5 μg) was analyzed by SDS-PAGE and stained using Coomassie brilliant blue, followed by (*B*) Western blotting using a mAb against ORP150. *C*, ORP150 and BSA were mixed with a ^{125}I-labeled peptide in a 50:1 peptide-to-protein molar ratio in sodium phosphate buffer. Samples were analyzed by SDS-PAGE and stained using Coomassie brilliant blue, followed by autoradiography of the stained gel.

Immunohistochemical analysis

After treatment of the preestablished TG3–2B tumor with the ORP150-survivin-2B peptide complex or PBS, tumor tissue was excised on day 35. The frozen tissues were stained with an anti-mouse CD4 mAb (Santa Cruz Biotechnology) or an anti-mouse CD8 mAb (Chemicon International) and then incubated with HRP-conjugated goat anti-rat Ig (Dako), followed by hematoxylin counterstaining. The numbers of tumor-infiltrating $CD4^+$ and $CD8^+$ T cells were counted in 10 high-power fields (HPF; ×400).

Statistical analysis

All experiments except for the tumor transplantation experiments were independently performed three times in triplicate. Results were given as means SEM. Comparisons between two groups were performed using Student's *t* test, whereas comparisons between multiple groups were done using ANOVA test, with a value of $p < 0.05$ considered to be statistically significant.

Results

Purification of secreted form of ORP150

Secreted ORP150 was purified from the culture supernatant of 293T-ORP4 cells using a Con A-Sepharose column as described in *Materials and Methods*. Fractions containing homogeneous ORP150 were collected and characterized by gel staining (Fig. 1*A*) and immunoblotting using a mAb to ORP150 (Fig. 1*B*). This revealed the homogeneity of ORP150 preparations with little contamination from other proteins. To critically evaluate the capacity of ORP150 to elicit immune responses, we carefully removed endotoxin that can activate innate immune responses. The purified ORP150 did not affect the cell surface expression of MHC class I molecules, MHC class II molecules, CD80, and CD86 of DCs, indicating the inability of ORP150 for DC maturation (data not shown). Additionally, DCs pulsed with the purified ORP150 did not stimulate the production of TNF-α or IL-12, also indicating minimal or no endotoxin in the purified ORP150 (data not shown).

Generation of ORP150-peptide complex in vitro

We ascertained and quantified the loading of peptides onto ORP150. We employed the iodinated SLC26 peptide as a tracer. As others and as we have already demonstrated, heat shock treatment accelerates the loading of peptides onto the binding sites of ORP150 and other HSPs (22). As shown in Fig. 1*C*, ORP150 bound labeled peptides efficiently, but control protein BSA bound

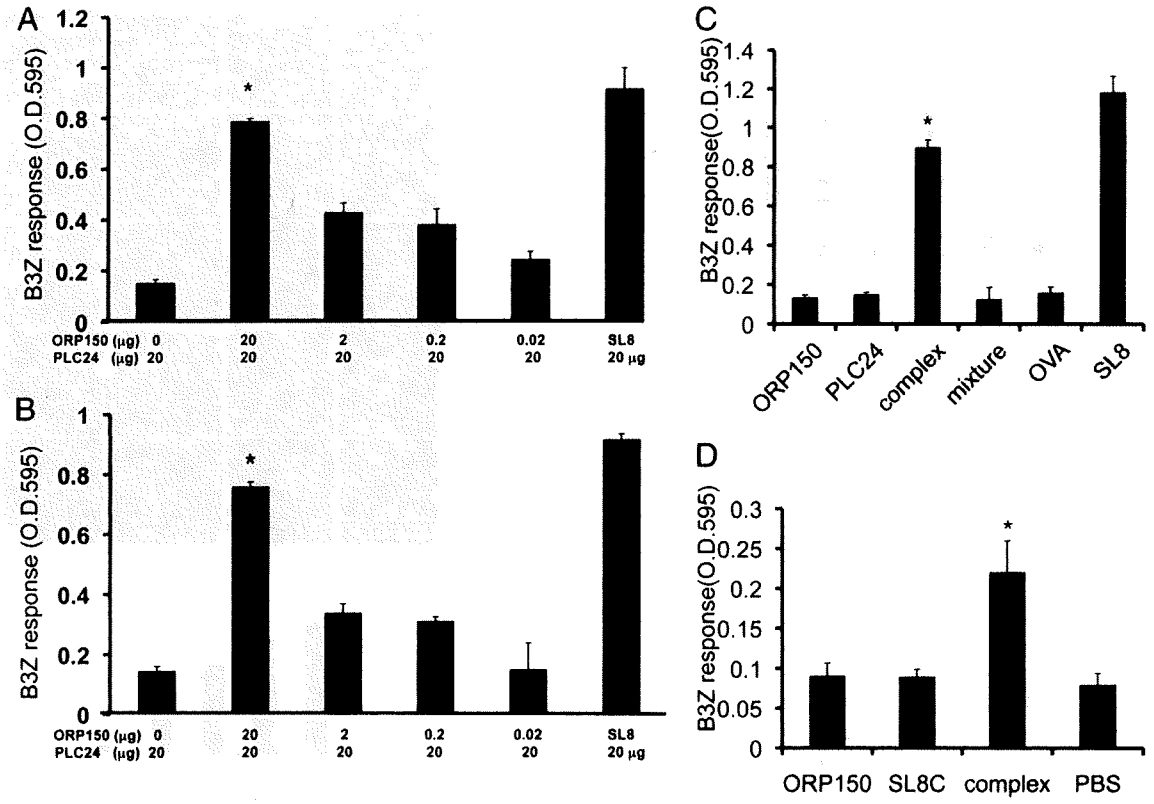

FIGURE 2. Cross-presentation of ORP150-chaperoned peptides by BMDCs in vitro and vivo. *A* and *B*, DCs (1×10^5) from C57BL/6 mice were pulsed with PLC24 (20 μg) alone, different ORP150/peptide dose ratios, or SL8 (1 μM) for 2 h at 37°C in 100 μl of Opti-MEM, and fixed for 1 min with 0.01% glutaraldehyde. *C*, ORP150 alone, PLC24 alone, a complex of both, a mixture of both, OVA, or SL8 was loaded onto BMDCs and cultured overnight with B3Z. The B3Z response was analyzed using CPRG. *D*, B6C3F1 mice were immunized into the footpads with ORP150 alone, SL8C alone, or a complex of both. After 12 h, $CD11c^+$ DCs were isolated from draining popliteal lymph nodes and cocultured with B3Z. The B3Z response was analyzed using CPRG. Data are shown as means ± SEM of three independent experiments. *, $p < 0.01$.

only marginal levels. The molar ratio of the bound SLC26 peptide to ORP150 was 3.95 mol of peptide per mole of ORP150 protein.

ORP150-PLC24 peptide complex is cross-presented by DCs in vitro and vivo

We first examined whether ORP150 facilitated the cross-presentation of the chaperoned precursor peptide. To determine the optimal ORP150/peptide dose ratio, DCs from C57BL/6 mice were loaded with different ORP150/peptide dose ratios for 2 h, fixed, and then cocultured with B3Z $CD8^+$ T cell hybridoma. As shown in Fig. 2, *A* and *B*, we observed that the complex generated using 20 μg/ml ORP150 and 20 μg/ml PLC24 peptide yielded sufficiently enough for the cross-presentation. In contrast, the same dose of PLC24 peptide alone was not cross-presented by DCs. Additionally, we examined whether complex formation between ORP150 and peptide was required for enhanced cross-presentation. DCs were pulsed with ORP150 alone, the PLC24 precursor peptide alone, a complex of both generated in vitro, a simple mixture of both, OVA protein, or SL8 peptide (for positive control) for 2 h at 37°C, then fixed, washed, and cultured with B3Z. The ORP150-PLC24 peptide complex elicited a strong B3Z response, while ORP150 alone, PLC24 peptide alone, or a simple mixture of both did not induce a B3Z response (Fig. 2*C*). Thus, the presence of ORP150 did not enhance the cross-presentation of uncomplexed PLC24 precursor peptide. These results indicated that cross-presentation of OVA-derived peptide was enhanced only if exogenous precursor peptide was complexed to the ORP150. Additionally, soluble OVA was not cross-presented at the concentration used (200 μg/ml) in this study (Fig. 2*C*). Furthermore, we examined whether cross-presentation of the ORP150-peptide complex occurred in vivo. We immunized B6C3F1 mice with the ORP150-SL8C complex, SL8C alone, or ORP150 alone and evaluated the appearance of $CD11c^+$ DCs that could stimulate B3Z in the draining lymph nodes (Fig. 2*D*). $CD11c^+$ DCs from mice immunized with the ORP150-SL8C complex elicited a significant B3Z response.

ORP150-peptide complex also facilitates Ag presentation to $CD4^+$ T cells

As the PLC24 peptide included both the $H\text{-}2K^b$ epitope SL8 and $I\text{-}A^k$ epitope PL19, we tested whether the ORP150-PLC24 peptide complex was presented through the MHC class II pathway, thereby inducing $CD4^+$ T cell responses. DCs from B6C3F1 mice were pulsed with ORP150 alone, PLC24 peptide alone, a complex of ORP150 and PLC24 peptide, a simple mixture of both, soluble OVA, or PL19 peptide (for positive control) for 2 h at 37°C, then cells were fixed, washed, and cocultured with KZO $CD4^+$ T cell hybridoma. While ORP150 alone and the PLC24 peptide alone were unable to induce $CD4^+$ T cell responses, the ORP150-PLC24 peptide complex induced a robust $CD4^+$ T cell response as well as a $CD8^+$ T cell response (Fig. 3). As expected, soluble OVA was also presented in association with $I\text{-}A^k$. These data indicated that a cross-presentation competent "ORP150-peptide complex" and incompetent "soluble OVA" might be translocated to different intracellular compartments for Ag processing and presentation. Based on our observation, we focused on the intracellular trafficking mechanism responsible for ORP150-mediated cross-presentation compared with soluble OVA.

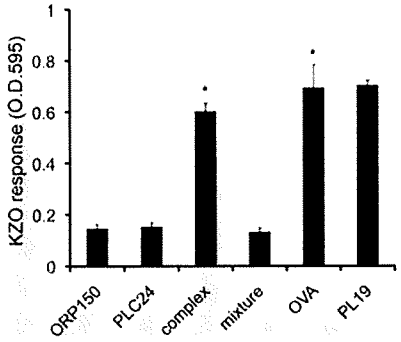

FIGURE 3. Ag presentation through MHC class II pathway in vitro. ORP150 alone, PLC24 alone, a complex of both, a mixture of both, OVA, or PL19 peptide was loaded onto BMDCs from B6C3F1 mice, which were cultured overnight with KZO. The KZO response was analyzed using CPRG. Data are shown as means ± SEM of three independent experiments. *, $p < 0.01$.

ORP150-PLC24 peptide complexes are cross-presented by a TAP-independent and recycling pathway

It is generally accepted that exogenous Ags are cross-presented by two distinct pathways in a TAP-dependent and -independent fashion. We examined whether cross-presentation of the ORP150-PLC24 peptide complex depended on TAP transport. DCs from B6C3F1 or TAP$^{-/-}$ mice were pulsed with the ORP150-PLC24 complex for 2 h. DCs from the TAP$^{-/-}$ mouse could process and present the ORP150-chaperoned peptide as efficiently as did DCs from the wild-type mouse (Fig. 4*A*). This suggested that ORP150-mediated cross-presentation might involve an endosome-recycling pathway. Therefore, we used primaquine, an inhibitor of membrane recycling, in the cross-presentation assay. DCs were preincubated with primaquine and subsequently pulsed with the ORP150-PLC24 complex. Primaquine did, indeed, show the dose-dependent inhibition of cross-presentation of the ORP150-chaperoned peptide (Fig. 4*B*). These data suggested that cross-presentation of the ORP150-PLC24 complex accessed recycling MHC class I molecules in endocytic compartments.

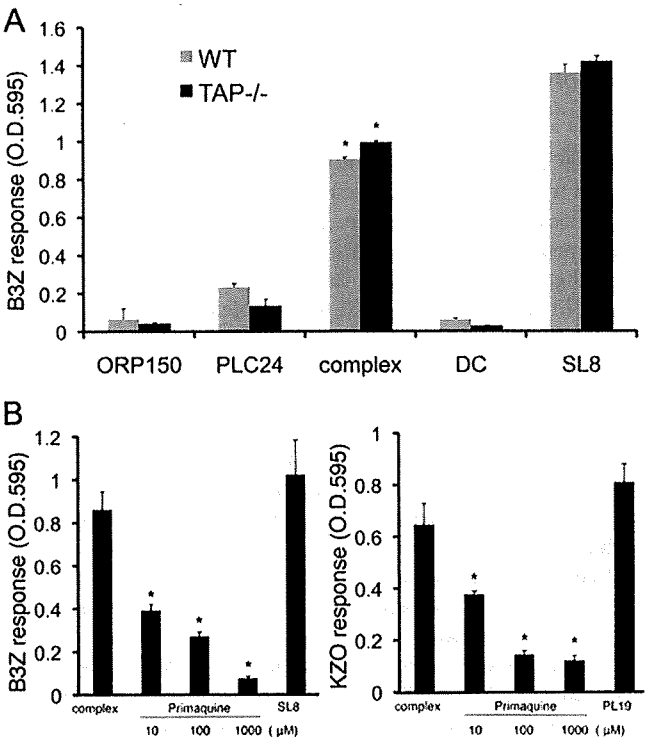

FIGURE 4. The ORP150-PLC24 peptide complex is cross-presented by TAP-independent pathway. *A*, BMDCs from B6C3F1 (wild type, WT) or TAP$^{-/-}$ mice were pulsed with ORP150 alone, PLC24 alone, a complex of both, or SL8. The DCs were then fixed, washed, and cultured overnight with B3Z. The B3Z response was analyzed using CPRG. *B*, DCs from B6C3F1 mice were preincubated with indicated concentrations of primaquine. The DCs were then pulsed with the ORP150-PLC24 complex or PL19 for 2 h. The B3Z response was analyzed using CPRG. Data are shown as means ± SEM of three independent experiments. *, $p < 0.01$.

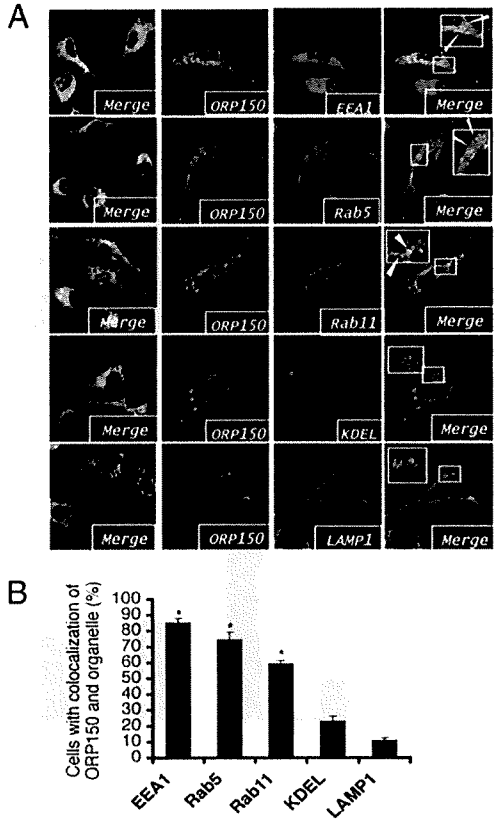

FIGURE 5. Intracellular localization of ORP150-PLC24 complex. *A*, BMDCs were incubated at 37°C with an Alexa Fluor 594-labeled ORP150-PLC24 complex for 1 h, then washed and fixed. Organelles were stained with an anti-EEA1 mAb for early endosomes, anti-Rab5 pAb for early endosomes, anti-Rab11 pAb for recycling endosomes, anti-KDEL mAb for ER, and anti-LAMP-1 pAb for late endosomes/lysosomes, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG or anti-mouse IgG and visualized with confocal laser microscopy. Colocalization of the internalized ORP150-peptide complex and each organelle is indicated with arrows. *B*, To quantify the percentage of the colocalization, a single *z*-plane of one cell was evaluated. For each protein and organelle combination, a total of 90 cells (30 cells from three independent experiments) were analyzed. Data are shown as means ± SEM of three independent experiments. *, $p < 0.01$.

Immunocytological localization of ORP150-PLC24 peptide complex

To further support all of the above results, we investigated the intracellular routing of ORP150 after uptake of it in DCs, using confocal laser microscopy. DCs were incubated with the Alexa Fluor 594-labeled ORP150-PLC24 peptide complex for 1 h. Following incubation, the cells were fixed and stained with Abs against markers for organelle structures such as EEA1, Rab5, LAMP-1, Rab11, and KDEL. The Alexa Fluor 594-labeled ORP150-peptide complex was detected in EEA1$^+$, Rab5$^+$-early endosomes and Rab11$^+$-recycling endosomes, but not in lysosomes or ER (Fig. 5*A*). Quantitative analysis of the colocalization between the exogenous ORP150-peptide complex and EEA1,

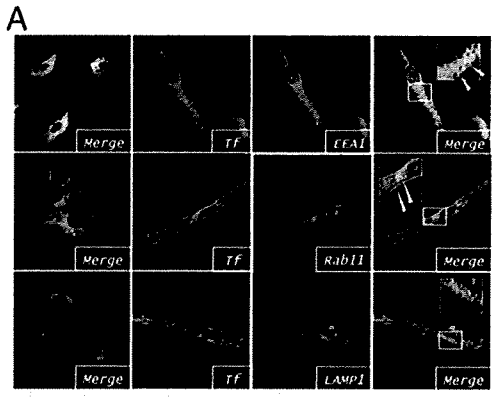

FIGURE 6. *A*, DCs were incubated at 37°C with Alexa Fluor 594-labeled transferrin (Tf). Organelles were stained with an anti-EEA1 mAb, anti-Rab5 pAb, anti-Rab11 pAb, and anti-LAMP-1 pAb, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG or anti-mouse IgG. Colocalization of internalized transferrin and each organelle is indicated with arrows. *B*, To quantify the percentage of the colocalization, a single *z*-plane of one cell was evaluated. For each protein and organelle combination, a total of 90 cells (30 cells from three independent experiments) were analyzed. Data are shown as means ± SEM of three independent experiments. *, $p < 0.01$.

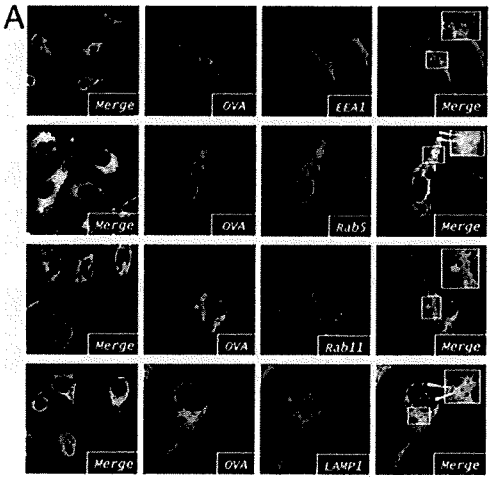

FIGURE 7. *A*, DCs were incubated at 37°C with Alexa Fluor 594-labeled OVA. Organelles were stained with an anti-EEA1 mAb, anti-Rab5 pAb, anti-Rab11 pAb, and anti-LAMP-1 pAb, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG or anti-mouse IgG. Colocalization of internalized OVA and each organelle is indicated with arrows. *B*, To quantify the percentage of the colocalization, a single *z*-plane of one cell was evaluated. For each protein and organelle combination, a total of 90 cells (30 cells from three independent experiments) were analyzed. Data are shown as means ± SEM of three independent experiments. *, $p < 0.01$.

Rab5, and Rab11 revealed an average colocalization incidence of 85.6, 74.4, and 58.9%, respectively, further evidencing that the exogenous ORP150-peptide complex was delivered to an endosome-recycling pathway (Fig. 5*B*). Moreover, we examined the dynamics of Alexa Fluor 594-labeled transferrin as a positive control protein for recycling endosomes (Fig. 6). As expected, transferrin localized to $EEA1^+$-static early endosomes and $Rab11^+$-recycling endosomes. In contrast, Alexa Fluor 594-labeled soluble OVA localized to the $Rab5^+$ early endosome as well as the $LAMP\text{-}1^+$ late endosome/lysosome, but not to the $EEA1^+$ or $Rab11^+$ compartment, thus indicating the dynamic endosomal pathway (Fig. 7). These results indicated that the ORP150-peptide complex was sorted into the static endosomal pathway, not the dynamic endosomal pathway. In contrast, the soluble OVA protein, which was not cross-presented, was translocated to the dynamic endosomal pathway. These data suggested that targeting to the static early endosome was required for efficient cross-presentation by DCs.

Early endosomes and recycling endosomes are the compartments where exogenous ORP150-chaperoned precursor peptides are processed and transferred onto recycling MHC class I molecules

To investigate in which compartment the ORP150-PLC24 peptide complex was processed and resulting peptide bound to MHC class I molecule, we used mAb 25D1.16, as this mAb recognized the SL8 peptide-H-$2K^b$ complex. We clearly observed that mAb 25D1.16 was detected in early endosome and recycling endosome, and not in the ER (Fig. 8). To confirm the staining specificity of mAb 25D1.16, DCs were incubated with ORP150-irrelevant peptide survivin-$2B_{80-88}$ complex and examined the colocalization of mAb 25D1.16 and each organelle. We did not observe the specific staining of mAb 25D1.16 (supplemental Fig. 1).[4] These data indicated that the ORP150-chaperoned precursor peptides were processed and bound to MHC class I within early endosomes and recycling endosomes, suggesting that recycling MHC class I molecules were necessary for efficient cross-presentation of the ORP150-chaperoned peptides.

Potent antitumor effect of immunization with ORP150-peptide complex against established tumor

HLA-A*2402/K^b transgenic mice are a well-established model for studying HLA-A*2402-restricted CTL epitopes and vaccine development (31). We previously reported survivin-2B as a universal tumor Ag and identified an HLA-A24-restricted antigenic peptide, survivin-$2B_{80-88}$ (AYACNTSTL), recognized by $CD8^+$ CTLs (32). We therefore examined the efficacy of ORP150-based immunotherapy using human tumor Ag survivin-2B as a surrogate Ag. HLA-A*2402/K^b transgenic mice were inoculated with methylchoranthrene-induced fibrosarcoma TG3 transfected with survivin-2B cDNA, TG3–2B. When tumor diameter reached 3–4 mm, treatment with s.c. injection of an ORP150-survivin-2B complex, ORP150 alone, survivin-2B peptide alone, or PBS was conducted (Fig. 9*A*). As shown in Fig. 9*B*, the growth of established TG3–2B tumors was significantly retarded in the group treated with the ORP150-survivin-2B complex compared with survivin-2B peptide alone, ORP150 alone, and PBS (vs survivin-2B, $p = 0.027$; vs ORP150, $p = 0.016$; vs PBS, $p = 0.0002$). We also

[4] The online version of this article contains supplemental material.

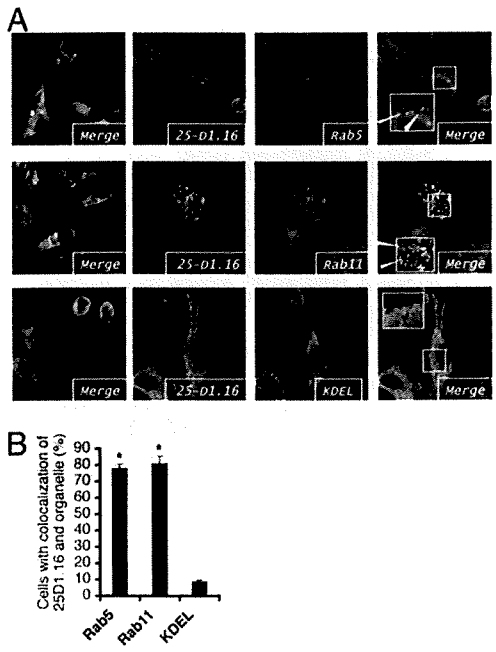

FIGURE 8. *A*, DCs were first incubated with ORP150 complexed with PLC24 for 1 h and fixed with cold acetone. DCs were then incubated with anti-mouse CD16/CD32 Fc-block to block nonspecific staining, followed by costaining with an Alexa Fluor 594-labeled mAb 25D1.16 and anti-organelle Abs conjugated with Alexa Fluor 488. Colocalization of H-2K^b/SL8 complex and each organelle are indicated with arrows. *B*, For evaluation of colocalization, a single z-plane of one cell was evaluated. For 25D1.16 and each organelle combination, a total of 90 cells (30 cells from three independent experiments) were analyzed. Data are shown as means ± SEM of three independent experiments. *, $p < 0.01$.

evaluated the tumor rejection rates for the ORP150-survivin-2B peptide complex and compared with survivin-2B peptide alone, ORP150 alone, and PBS (vs survivin-2B, $p = 0.070$; vs ORP150, $p = 0.048$; vs PBS, $p = 0.007$) (Fig. 9*C*). Notably, six of nine mice in the ORP150-survivin-2B complex-treated group rejected the established tumors. These data strongly suggested that immunization with the ORP150-tumor peptide complexes were an effective immunotherapeutic approach for various types of cancer expressing survivin-2B and HLA-A*2402. Next, we confirmed whether CD8$^+$ T cells infiltrated within the established tumor mass in response to immunization with the ORP150-survivin-2B complex by immunohistological analysis (Fig. 9*D*). More CD8$^+$ T cells were observed in the tumor tissues injected with complex (328 cells/10 HPF) than in the PBS control (59 cells/10 HPF). We also examined CD4$^+$ T cell infiltration. The extent of CD4$^+$ T cell infiltration in mice immunized with the ORP150-survivin-2B complex (176 cells/10 HPF) was also greater than that of mice immunized with PBS (53 cells/10 HPF). Moreover, we tested whether in mice treated with the ORP150-survivin-2B complex, survivin-2B-specific CTL responses were induced. Spleen cells of mice treated with the ORP150-survivin-2B complex showed significant cytotoxicity against TG3–2B cells and survivin-2B-coated RMA-S-A*2402 cells, but not survivin-2B-noncoated RMA-S-A*2402 cells or YAC-1 cells (Fig. 9*E*). We also examined the tetramer assay using survivin-2B-HLA-A*2402/human β_2-microglobulin tetramer (33). However, we were unable to detect apparent tetramer-positive population (data not shown). The reason for this may be attributed to the species difference of β_2-microglobulin. These results showed that the ORP150-Ag peptide complex induced a strong CTL response to the chaperoned peptide and that this response was sufficiently strong to generate antitumor effects.

Discussion

It is well demonstrated that immunization with tumor-derived HSPs or HSPs complexed with an Ag peptide/protein elicits tumor- or Ag-specific CD8$^+$ T cell responses. Above all, it has been shown that Hsp70- and gp96-Ag complexes facilitate Ag presentation in association with MHC class I molecules (16, 34, 35). However, the ability of HSPs to facilitate the presentation of MHC class II-restricted epitopes and to prime CD4$^+$ T cells has been relatively unexplored. In recent reports, it has been shown that Hsp70- and gp96-Ag complexes facilitate Ag presentation in association with both MHC class I and class II molecules (34, 36, 37). In this study, we demonstrated that ORP150-Ag complexes also facilitated Ag presentation in association with both MHC class I molecules and MHC class II molecules via an endosome-recycling pathway. In vaccine development, ORP150 has advantages for the induction of specific CTLs due to the simultaneous activation of specific helper T cells, which are required for efficient

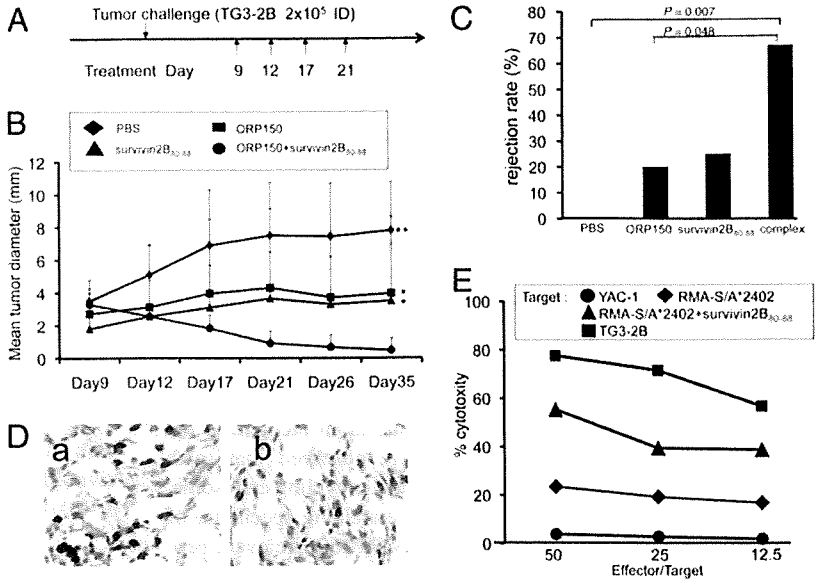

FIGURE 9. ORP150-tumor Ag peptide complexes induce strong antitumor effects. *A*, The protocol for immunotherapy is shown. *B*, A total of 2×10^5 TG3–2B cells were first injected intradermally into HLA-A*2402/K^b mice (10 animals/group). When mean tumor diameter reached 3–4 mm, mice were given the treatment with the ORP150 (50 μg)-survivin-2B$_{80-88}$ (50 μg) complex, ORP150 (50 μg) alone, survivin-2B$_{80-88}$ (50 μg) alone, or PBS twice a week. *C*, The remaining 8–10 mice in each group were observed for the tumor rejection rate. *D*, The frozen tissues excised from mice treated with (*a*) the ORP150-survivin-2B$_{80-88}$ peptide complex or (*b*) PBS control was stained with an anti-mouse CD8 mAb (×400). *E*, Immunization with the ORP150-Ag peptide complex induced peptide specific CTLs. After immunizaton with the ORP150-survivin-2B$_{80-88}$ peptide complex, splenocytes of the mice were cultured with survivin-2B$_{80-88}$ peptide for 5 days and tested for cytotoxicity. Representative data are shown as means ± SEM of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$.

CTL induction. In other words, when it is required for activation of $CD8^+$ T cells as well as $CD4^+$ T cells via Ag presentation in the context of both MHC class I and class II molecules, ORP150 can be a potent enhancer in immunotherapy.

Recent studies have revealed that stressful stimuli induce active release of intracellular HSPs into the extracellular milieu. The extracellular HSPs play an important role in initiating immune responses against microbial infection and neoplastic cells (10, 38). Because virus-infected cells and tumor cells are not able to prime naive $CD8^+$ T cells due to the lack of costimulatory molecules, priming of $CD8^+$ T cells against tumor cells and virus-infected cells requires cross-presentation by APCs. Extracellular HSP-Ag complexes, which are released from damaged tumor cells or virus-infected cells, are considered to be candidate Ag sources for cross-presentation. The pathway for cross-presentation has been shown to be comprised of two distinct intracellular routes, a proteasome-TAP-dependent pathway and an endosome-recycling pathway (22, 39). Recent reports have identified the pathway wherein peptide exchange onto recycling MHC class I molecules occurs within early endosomal compartments (40). We have shown that ORP150-mediated cross-presentation is independent of TAP and sensitive to primaquine, indicating that sorting of peptides onto MHC class I occurs via an endosome-recycling pathway. Very recently, Lakadamyali et al. (29) have shown that early endosomes are comprised of two distinct populations: a dynamic population that is highly mobile on microtubules and matures rapidly toward the late endosome, and a static population that matures much more slowly. Cargos destined for degradation, including low density lipoproteins, epidermal growth factors, and influenza virus, are internalized and targeted to the $Rab5^+$, $EEA1^-$-dynamic population of early endosomes, thereafter trafficking to $Rab7^+$-late endosmes. In contrast, the recycling ligand transferrin is delivered to $Rab5^+$, $EEA1^+$-static early endosomes, followed by translocating to $Rab11^+$-recycling endosomes. Additionally, Burgdorf et al. clearly demonstrated that a mannose receptor introduced OVA specifically into an $EEA\text{-}1^+$, $Rab5^+$-stable early endosomal compartment for subsequent cross-presentation (30). In contrast, pinocytosis conveyed OVA to lysosomes for class II presentation. Of interest, OVA endocytosed by a scavenger receptor did not colocalize with EEA1, but colocalized with LAMP-1 in lysosome, leading to presentation in the context of MHC class II molecules. We showed that the ORP150-peptide complex is targeted into $Rab5^+$, $EEA1^+$-early endosome after internalization by DCs, suggesting that preferential sorting to the static endosome is necessary for cross-presentation of ORP150-peptide complexes. In contrast, soluble OVA protein, which was not cross-presented, targeted to the $EEA1^-$ and $LAMP\text{-}1^+$-dynamic early endosome-late endosome/lysosome pathway, leading to degradation and presentation in the context of MHC class II molecules. These data suggested that ORP150 shuttled the chaperoned precursor peptide into the static endosome-recycling pathway, preventing further degradation, followed by transferring the peptide onto recycling MHC class I molecules. If the ORP150-peptide complexes were loaded into late endosomes/lysosomes, they might be quickly degraded. Therefore, we consider that it is necessary that the ORP150-chaperoned peptide complex targets the early endosome and is processed in the static endosome-recycling pathway.

We have shown that immunization with ORP150-peptide complexes elicits strong CTL responses and antitumor effects. In contrast, we have reported that Hsp70-peptide complex, which is a representative member of Hsp70 superfamily, elicits only weak CTL responses (22). This may be because ORP150 is more efficient in binding peptides than is Hsp70. The ability of ORP150 to bind polypeptides better than other Hsp70 family members is largely due to its enlarged C-terminal helical domain (41, 42). Moreover, it may help that peptide binding to ORP150 is independent of ATP, which is in contrast with ATP-dependent substrate binding of other Hsp70 family members (42).

Finally, to date, SR-A and SREC-I have been identified as receptors for ORP150 expressed on APCs (20). However, whether SR-A or SREC-I is responsible for the efficient cross-presentation by DCs remains unclear. Such receptors should introduce the ORP150-peptide complex specifically into a static early endosomal compartment for the subsequent cross-presentation. Sorting in the endocytic system is a complex and highly dynamic process in which a wide variety of sorting motifs are recognized by specific sorting machinery to direct the membrane protein such as a receptor to its destination. Namely, after the receptor-mediated endocytosis, sorting of the ORP150-Ag complex into the static early endosome may be regulated by specific sorting motifs. The ORP-specific receptor might bear the sorting motifs, which is responsible for the trafficking ORP150 receptor-ORP150 complex to the static early endosomes. To elucidate the mechanism for sorting ORP150 into these compartments, the specific receptors for the ORP150-mediated cross-presentation should be defined. Moreover, it is very important to identify the motifs intrinsic in such receptors that govern static early endosomal sorting. Identification and characterization of the cross-presentation-responsible receptor and its role as an efficient sorter to the static early endosome have important implications in vaccine development strategies. We are currently investigating the ORP150-specific receptors expressed on APCs responsible for the cross-presentation.

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Disclosures

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Side population cells have the characteristics of cancer stem-like cells/cancer-initiating cells in bone sarcomas

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BACKGROUND: Several human cancers have been found to contain cancer stem-like cells (CSCs) having cancer-initiating ability. However, only a few reports have shown the existence of CSCs in bone and soft tissue sarcomas. In this study, we identified and characterised side population (SP) cells that showed drug-resistant features in human bone sarcoma cell lines.

METHODS: In seven osteosarcoma cell lines (OS2000, KIKU, NY, Huo9, HOS, U2OS and Saos2) and in one bone malignant fibrous histiocytoma (MFH) cell line (MFH2003), the frequency of SP cells was analysed. Tumourigenicity of SP cells was assessed *in vitro* and *in vivo*. Gene profiles of SP cells and other populations (main population; MP) of cells were characterised using cDNA microarrays.

RESULTS: SP cells were found in NY (0.31%) and MFH2003 (5.28%). SP cells of MFH2003 formed spherical colonies and re-populated into SP and MP cells. In an NOD/SCID mice xenograft model, 1×10^3 sorted SP cell-induced tumourigenesis. cDNA microarray analysis showed that 23 genes were upregulated in SP cells.

CONCLUSIONS: We showed that SP cells existed in bone sarcoma cell lines. SP cells of MFH2003 had cancer-initiating ability *in vitro* and *in vivo*. The gene profiles of SP cells could serve as candidate markers for CSCs in bone sarcomas.

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Over the past three decades, there have been remarkable advances in the treatment of bone and soft tissue sarcomas. These include the introduction of adjuvant chemotherapy, establishment of guidelines for adequate surgical margins and the development of post-excision reconstruction (Fletcher *et al*, 2002; Lewis, 2007). However, the prognosis of non-responders to chemotherapy is still poor and the mechanisms of tumourigenesis of bone and soft tissue sarcomas remain to be demonstrated.

Generally, cancer masses are considered to be a complex of heterogeneous but equally malignant cell populations. However, recent stem cell research on the development of normal organs has drawn attention to the existence of a 'cancer stem-like cell (CSC)' counterpart, which is characterised by its self-renewal capacity, differentiation potential, and cancer-initiating ability (Visvader and Lindeman, 2008). On the basis of these characteristics, CSCs have been postulated to be responsible for driving the growth of tumours and for the recurrence of neoplasms after current therapeutic modalities are used.

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Initial attempts to characterise CSCs were accomplished using cell surface molecules in acute myeloid leukaemia. Several groups that found CSCs capable of initiating leukaemia were found in the $CD34^+CD38^-$ fraction (Warner *et al*, 2004). Recently, CSCs have been isolated from several human solid tumours that have markers for putative normal stem cells, including breast cancer ($CD44^+CD24^-ESA^+$) (Al-Hajj *et al*, 2003), pancreatic cancer ($CD44^+CD24^-ESA^+$, $CD133^+CXCR4^+$) (Hermann *et al*, 2007; Li *et al*, 2007), brain cancer ($CD133^+$) (Singh *et al*, 2004), prostate cancer ($CD44^+/\alpha_2\beta_1^{hi}/CD133^+$) (Collins *et al*, 2005), hepatocellular carcinoma ($CD133^+$) (Yin *et al*, 2007) and colon cancer ($CD133^+$) (Ricci-Vitiani *et al*, 2007).

On the other hand, in the analysis of haematopoietic stem cells, a sub-population that effluxes the DNA-binding dye Hoechst 33342 out of the cell membrane through an ATP-binding cassette (ABC) transporter was recognised as a stem cell population (Goodell *et al*, 1996; Zhou *et al*, 2002; Robinson *et al*, 2005). This cell population expressing the ABC transporter was defined as side population (SP) cells, which were distinguished from cells of the other population (main population; MP). Recent studies demonstrated that SP cells could be characterised as CSCs in primary tissues of gastrointestinal cancers (Haraguchi *et al*, 2006) and ovarian cancer (Szotek *et al*, 2006). SP cells were also shown in established tumour cell lines with different origins, such as glioma (Kondo *et al*, 2004), breast (Kruger *et al*, 2006) and thyroid cancer monoclonal cell lines (Mitsutake *et al*, 2007).