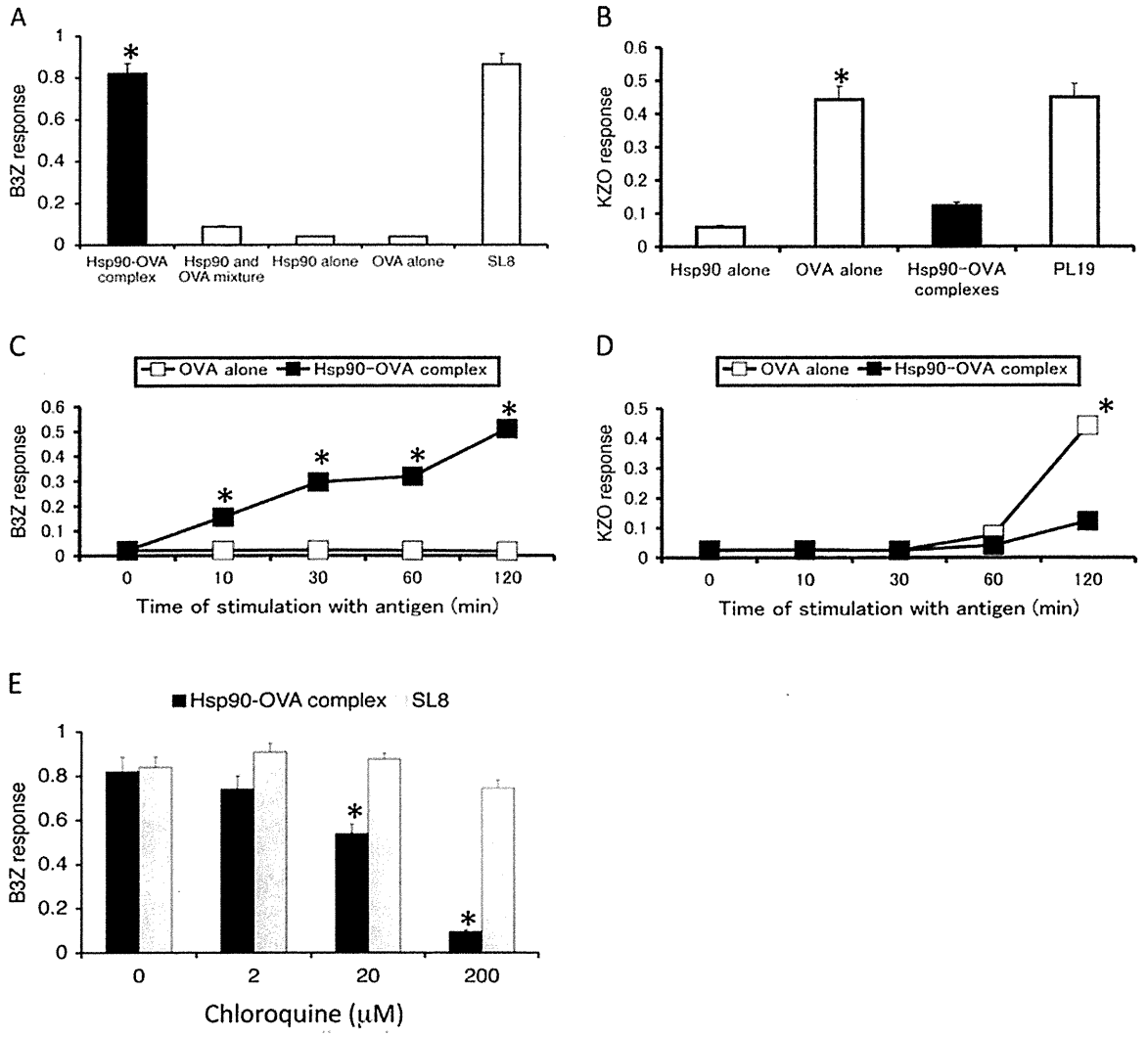

Fig. 4. Hsp90–OVA complex is presented selectively and preferentially through MHC class I pathway. (A) DCs were pulsed with an Hsp90 (200 μg ml ^{1})–OVA (200 μg ml^{-1}) complex, a simple mixture of the two, Hsp90 alone (200 μg ml^{-1}), OVA alone (200 μg ml^{-1}) or SL8 (1 μM) for 2 h at 37°C, and then fixed, washed and cultured overnight with B3Z. The B3Z response was measured as β-galactosidase activity using CPRG by the absorbance at 595 nm. SL8 served as a positive control. (B) DCs were pulsed with Hsp90 alone, OVA alone, Hsp90–OVA or PL19 (1 μM) for 2 h at 37°C, and then fixed, washed and cultured overnight with KZO. PL19 served as a positive control. (C and D) DCs were pulsed with OVA alone or Hsp90–OVA at 37°C, harvested at different time points between 10 min and 2 h, and then fixed, washed and cultured overnight with B3Z (C) or KZO (D). (E) DCs were pre-incubated with chloroquine at 37°C for 2 h, and then pulsed with Hsp90–OVA or SL8 for 2 h. The DCs were then fixed, washed and cultured overnight with B3Z. Data are shown as means ± SD of three independent experiments. *, $P < 0.01$.

used cycloheximide, an inhibitor of protein synthesis, and primaquine, an inhibitor of membrane recycling. DCs were pre-incubated with cycloheximide or primaquine and subsequently pulsed with Hsp90–OVA. Both inhibitors decreased cross-presentation of the complex dose-dependently (Fig. 5B and C). These data suggested that cross-presentation of Hsp90–OVA accessed both newly synthesized MHC class I molecules in ER and recycling MHC class I molecules in endocytic compartments.

Both proteasomal processing and proteolysis by cysteine/serine proteases are involved in cross-presentation of Hsp90–OVA complex

Proteins are degraded by proteasomes into shorter peptides via the TAP-dependent pathway of cross-presentation. To test whether proteasomal enzymes were involved in cross-presentation of Hsp90–OVA, we used MG-132, a potent proteasome inhibitor. MG-132 partially blocked cross-presentation of the complex but had no substantial effect on SL8 presentation (Fig. 5D). Therefore, proteasomal proteolysis is required for the TAP-dependent pathway of the Hsp90–OVA complex.

A recent report indicated that the TAP-independent pathway of cross-presentation depended on cysteine/serine proteases in endocytic compartments. We tested the effect of treating DCs with leupeptin, an inhibitor of cysteine and serine proteases. Leupeptin partially inhibited the DC cross-presentation capacity of the Hsp90–OVA complex (Fig. 5E). As expectedly, presentation of soluble OVA by MHC class II was completely inhibited by leupeptin pretreatment (Fig. 5F). Notably, cross-presentation of Hsp90–OVA was further inhibited by treatment with a combination

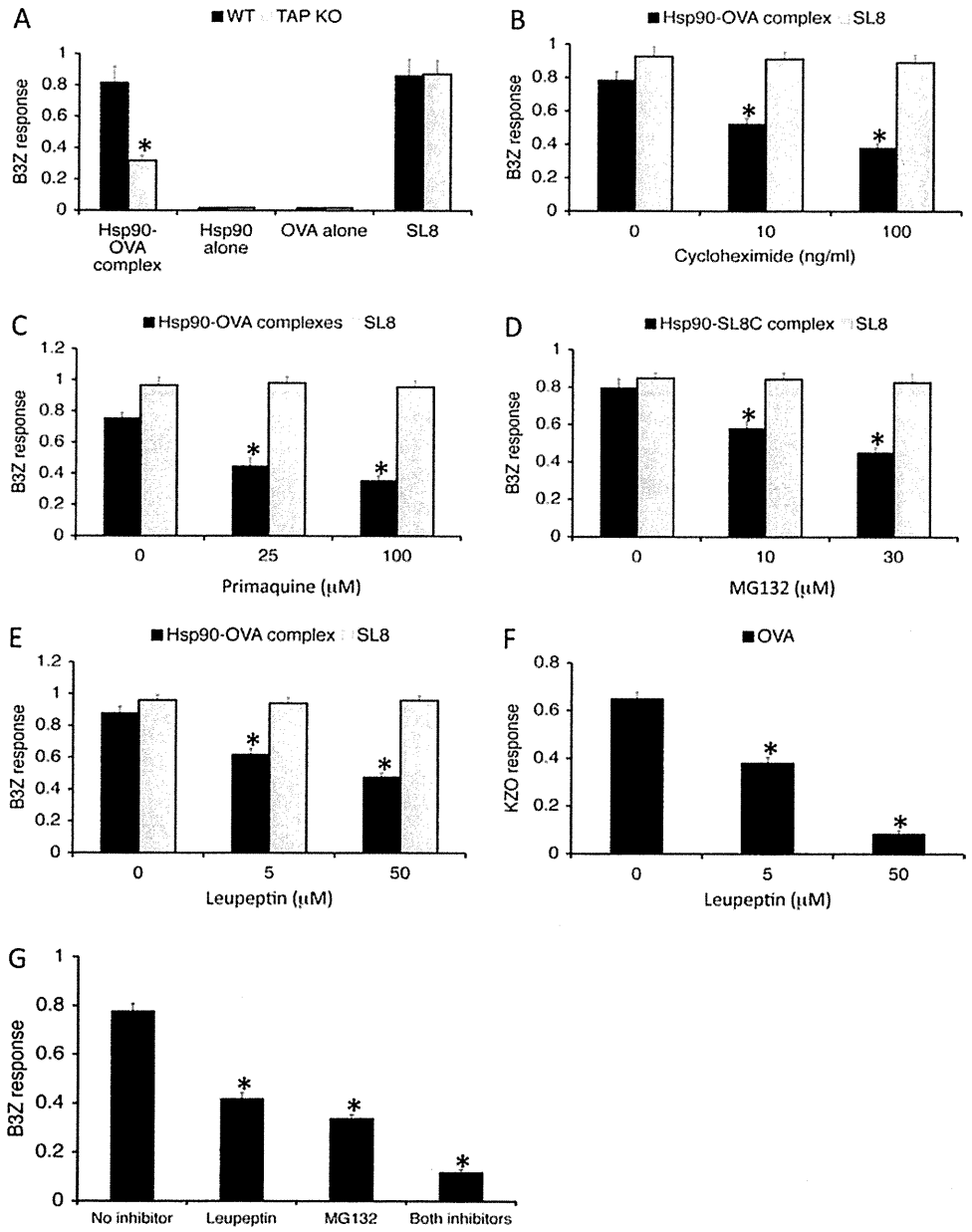

Fig. 5. Hsp90–OVA complex is cross-presented by both TAP-dependent and TAP-independent pathways. (A) DCs from B6C3F1 (WT) or TAP$^{-/-}$ (TAP KO) mice were pulsed with Hsp90 alone (200 μg ml^{-1}), OVA alone (200 μg ml^{-1}), Hsp90–OVA or SL8 (1 μM) for 2 h at 37°C. The DCs were then fixed, washed and cultured overnight with B3Z. The B3Z response was measured as β-galactosidase activity using CPRG by the absorbance at 595 nm. SL8 served as a positive control. (B and C) DCs were pre-incubated with cycloheximide (B) or primaquine (C) at 37°C for 2 h, and then pulsed with Hsp90–OVA or SL8 for 2 h. The DCs were then fixed, washed and cultured overnight with B3Z. The B3Z response was measured by the absorbance at 595 nm. (D–G) DCs were pre-incubated with MG-132 (D), leupeptin (E and F) or both of them (G) at 37°C for 2 h, and then pulsed with Hsp90–OVA, SL8 or OVA for 2 h. The DCs were then fixed, washed and cultured overnight with B3Z or KZO. The B3Z or KZO response was measured by the absorbance at 595 nm. Data are shown as means ± SD of three independent experiments. *, $P < 0.01$.

of MG-132 (30 μM) and leupeptin (50 μM) (Fig. 5G). These findings indicated that Hsp90–OVA was cross-presented by two distinct proteolytic pathways, one involving proteasomes and the other involving endocytic compartments.

Exogenous OVA and Hsp90–OVA complex show different subcellular localization after uptake by DCs

To further support all of the above results, we investigated the fate of Hsp90–OVA after its uptake in DCs, using confocal laser microscopy. DCs were incubated with Alexa 594-labeled soluble OVA or the Hsp90–Alexa 594-labeled–OVA complex for 1 h. Following incubation, the cells were fixed and stained with antibodies against markers for organelle structures such as Rab5, LAMP-1, Rab11, KDEL and LMP-2. Soluble OVA was detected in early endosomes to lysosomes, but not in recycling endosomes, ER or proteasomes (Fig. 6A). On the other hand, Hsp90-chaperoned Alexa 594-labeled OVA was observed to co-localize with

early endosomes, recycling endosomes and proteasomes, but not in lysosomes or ER (Fig. 6B). To confirm whether exogenous Hsp90 also co-localized with cytosolic proteasome, DCs were incubated with Alexa 594-labeled Hsp90 complexed with OVA. We observed that the exogenous Hsp90 was detected in early endosomes and proteasome subunit LMP-2, but not in lysosomes and recycling endosomes (Fig. 7). These data demonstrated that the part of exogenous Hsp90–OVA complex translocated from early endosomes into the cytosol while it has been unclear whether the Hsp90 and OVA remain in the complex or not, followed by association with proteasomes. Moreover, unlike in the data from localization of Hsp90–Alexa 594-labeled OVA, Alexa 594-labeled Hsp90 did not co-localize with recycling

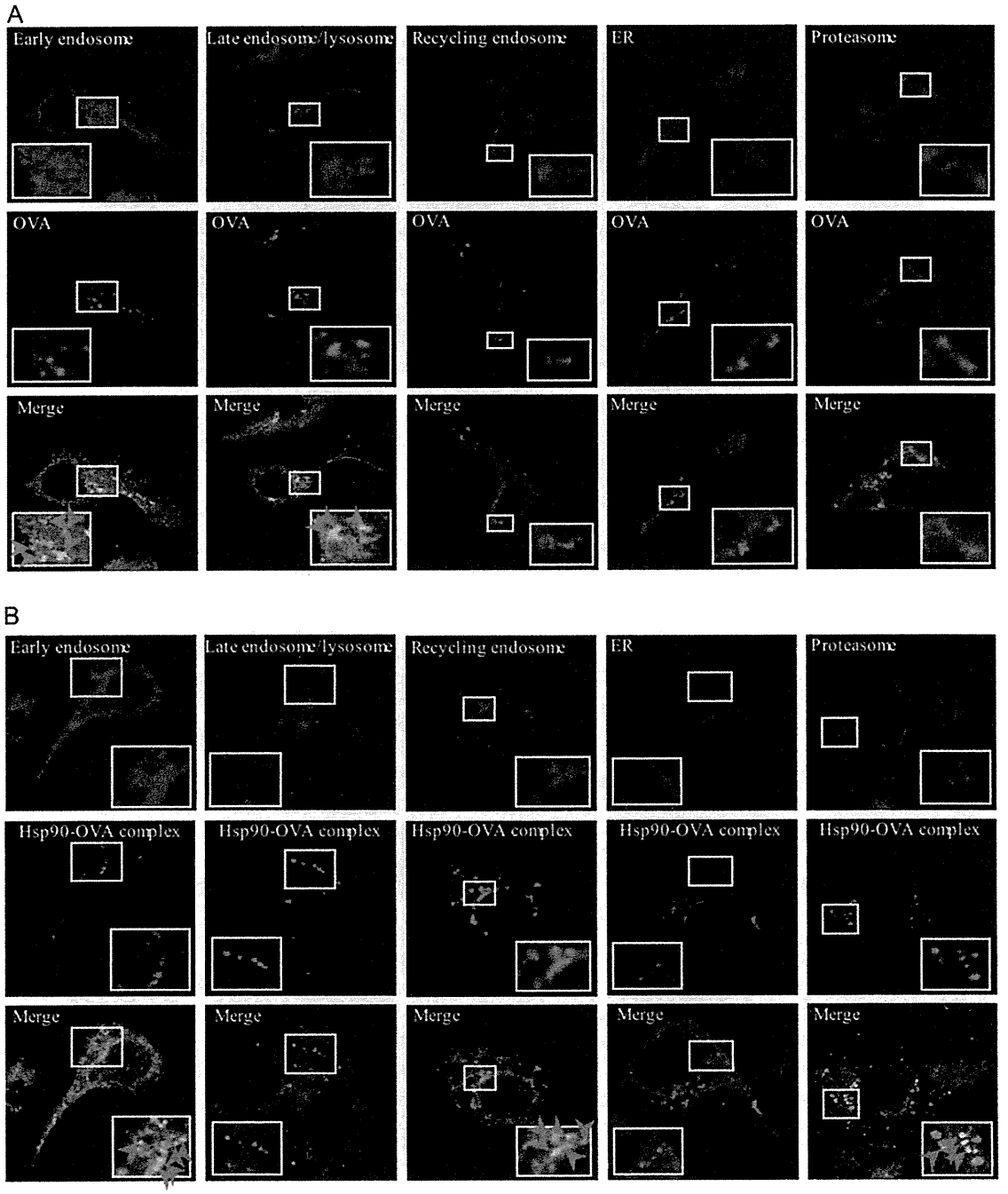

Fig. 6. Exogenous OVA and Hsp90–OVA complex show different subcellular localization after uptake by DCs. Immunocytological localization of OVA and Hsp90–OVA complex. (A, B) DCs were incubated at 37°C with Alexa Fluor 594-labeled OVA (A), Hsp90–Alexa 594-labeled OVA (B) for 1 h, and then washed and fixed. Organelles were stained with an anti-Rab5 pAb for early endosomes, anti-LAMP-1 pAb for late endosomes/lysosomes, anti-Rab11 pAb for recycling endosomes, anti-KDEL mAb for ER and anti-LMP2 pAb for proteasomes, conjugated with Alexa Fluor 488 and visualized with confocal laser microscopy (original magnification ×630). Co-localization of internalized OVA or Hsp90–OVA and each organelle is indicated with arrows. Data are representative of three independent experiments.

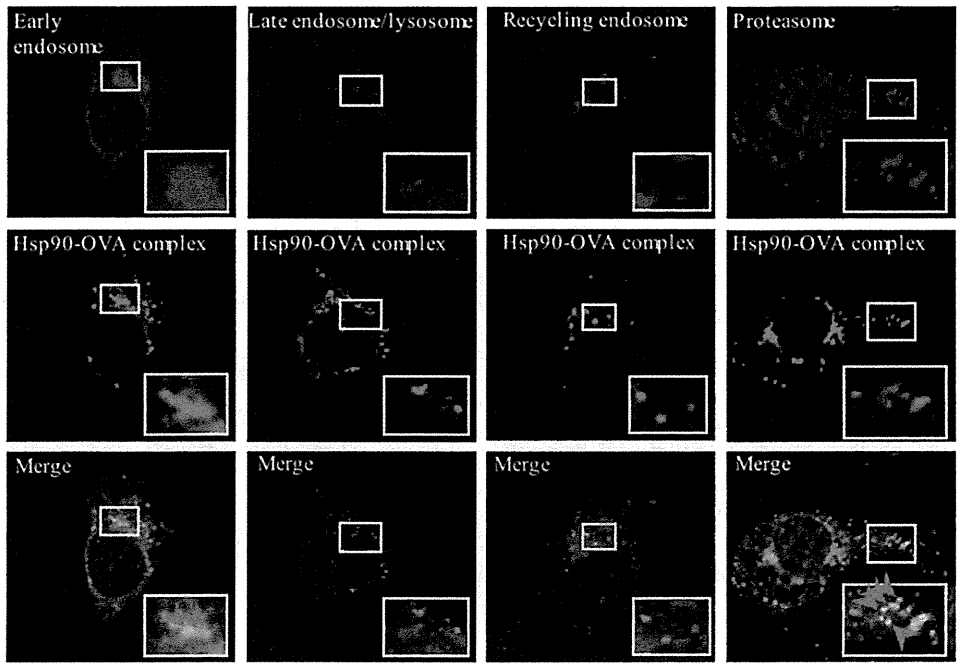

Fig. 7. Exogenous Hsp90 co-localized with cytosolic proteasome. DCs were incubated with Alexa Fluor 594-labeled Hsp90 complexed with OVA for 1 h. Organelles were stained with an anti-Rab5 pAb, anti-LAMP-1 pAb, anti-Rab11 pAb and anti-LMP2 pAb, conjugated with Alexa Fluor 488 and visualized with confocal laser microscopy (original magnification ×630). Co-localization of internalized OVA or Hsp90–OVA and each organelle is indicated with arrows. Data are representative of three independent experiments.

endosomes, suggesting that a part of OVA chaperoned by Hsp90 was released from Hsp90 within early endosomes, followed by translocation to recycling endosomes where recycling MHC class I molecules existed.

Inhibition of endogenous Hsp90 does not affect the cross-presentation of exogenous Hsp90–OVA complex

Recently, it has been demonstrated that endogenous cytosolic Hsp90 plays an important role in antigen presentation of exogenous as well as endogenous antigens. To investigate whether endogenous Hsp90 was responsible for the cross-presentation of the exogenous Hsp90–OVA complex, we treated DCs with the Hsp90-specific inhibitor radicicol in a cross-presentation assay. The results showed that treatment of DCs with radicicol did not affect the cross-presentation of exogenous Hsp90–OVA, suggesting that endogenous Hsp90 might not responsible for the exogenous Hsp90-mediated cross-presentation (Fig. 8A). Moreover, radicicol treatment did not affect the co-localization of the internalized Hsp90-chaperoned OVA and LMP2 (Fig. 8B). In contrast, high concentration of soluble OVA (200 mg ml^{-1}) was cross-presented by DCs, although it was considerably less efficiently than Hsp90–OVA complex. Interestingly, this cross-presentation was radicicol sensitive, indicating that endogenous Hsp90 played a very important role in the cross-presentation of exogenous OVA (Fig. 8C). However, as it is not clear how endogenous Hsp90 participates in the cross-presentation, we are currently studying the possibility that endocytosed soluble OVA might be translocated by endogenous Hsp90 into the cytosol for proteasomal degradation. It has been demonstrated that treatment with radicicol induces stress responses such as induction of Hsp72 protein. Therefore, we examined whether the concentration of radicicol used in our experiments was effective to induce Hsp72 expression. When DCs were exposed to the radicicol at concentrations of 1.0 μM and 5.0 μM for 2 h, the expression of Hsp72 was clearly induced compared with control cells (Fig. 8D), indicating that concentration of radicicol used in the cross-presentation assay was sufficient enough for the inhibition of Hsp90 function of DCs. Thus, collectively, exogenous Hsp90, but not endogenous Hsp90, might be required for cross-presentation of exogenous antigens chaperoned by Hsp90.

Exogenous Hsp90 contributes to the translocation of chaperoned OVA to proteasomes

We hypothesized that exogenous Hsp90 itself was responsible for the efficient translocation of chaperoned OVA to the site for proteasomal degradation. To test this idea, we introduced an anti-Hsp90 antibody into the cytosol of DCs to inhibit the activities of both endogenous Hsp90 and exogenous Hsp90. We utilized the BioPORTER protein delivery reagent to introduce the anti-Hsp90 mAb into DCs. The BioPORTER/antibody complexes are endocytosed by the cells and then fuse with the endosome releasing the BioPORTER-captured antibody into the cytoplasm. To examine the localization of the transduced antibody, FITC-antibody control protein was mixed with the BioPORTER reagent and added to DCs. After incubation for 4 h, FITC-antibody control protein was mixed with the BioPORTER reagent and added to DCs. After incubation for 4 h, the cells were washed and fixed. Organelles were stained with an anti-Rab5 pAb for early endosomes conjugated with Alexa 594 and visualized with confocal laser microscopy (Fig. 9A). The results showed that the transduced antibody localized in cytosol but not

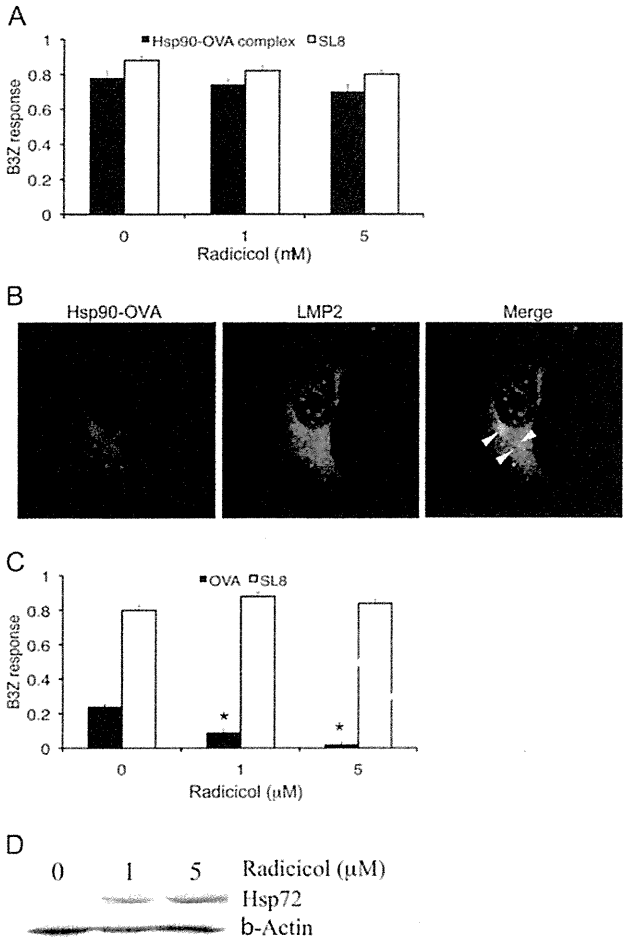

Fig. 8. Inhibition of endogenous Hsp90 does not affect the cross-presentation of exogenous Hsp90–OVA complex. (A) DCs were pre-incubated with radicicol at the indicated doses. Two hours after pre-incubation, the DCs were pulsed with Hsp90 (200 μg ml^{-1})–OVA (200 μg ml^{-1}) or SL8 (1 μM) for 1 h, and fixed, washed and cultured overnight with B3Z. The B3Z response was measured by the absorbance at 595 nm. (B) DCs were pre-incubated with radicicol at 5 μM. Two hours after pre-incubation, DCs were incubated at 37°C with Hsp90–Alexa Fluor 594-labeled–OVA complex for 1 h, and then washed and fixed. Organelles were stained with an anti-LMP2 pAb conjugated with Alexa Fluor 488 and visualized with confocal laser microscopy. Co-localization of internalized Hsp90–OVA and LMP2 is indicated with arrows. (C) DCs were pre-incubated with radicicol at the indicated doses. Two hours after pre-incubation, the DCs were pulsed with OVA (200 mg ml^{-1}) or SL8 (1 μM) for 1 h, fixed, washed and cultured overnight with B3Z. The B3Z response was measured by the absorbance at 595 nm. (D) Following treatment of DCs with the indicated concentrations of radicicol for 2 h and culture for 24 h, the cell lysate was harvested. Western blotting of the expression of Hsp72 was done using a specific antibody. The ß-actin level was used as a loading control. Data are shown as means ± SD of three independent experiments. *, $p < 0.01$.

endosomes, suggesting that the transduced anti-Hsp90 antibody could be expected to bind to both exogenous and endogenous Hsp90 in the cytosol. In fact, the transduced Alexa 594-labeled anti-Hsp90 mAb was co-localized with Alexa 488-labeled exogenous Hsp90 within DCs (data not shown). We next examined whether transduction of the anti-Hsp90 mAb affected the intracellular localization of the exogenous Hsp90–OVA complex in DCs by confocal laser microscopy. We observed that when the anti-Hsp90 mAb was transduced into the cytosol of DCs, co-localization of Hsp90–Alexa 594–OVA complex and Alexa 488-labeled proteasome subunit LMP2 was poorly detected. Although it should be elucidated how the transduced antibody affected the Hsp90 activity, these data suggested that transduction of the anti-Hsp90 antibody resulted in marked inhibition of transportation of Hsp90-chaperoned OVA to the proteasome (Fig. 9B). In agreement with these data, transduction of the anti-Hsp90 antibody into DCs, but not the control antibody, resulted in partial inhibition of Hsp90-mediated cross-presentation (Fig. 9C). In contrast, B3Z responses to DCs pulsed with the SL8 peptide with or without antibody transduction were not affected. Since Hsp90-mediated cross-presentation was involved in both the cytosolic and endosomal pathways, this partial inhibition seemed to reflect the inhibition of a cytosolic–proteasome–TAP-dependent pathway, whereas the endosomal pathway might not have been affected. Therefore, we tested whether the transduction of the anti-Hsp90 mAb plus leupeptin treatment of DCs abolished the cross-presentation of the Hsp90–OVA complex. The results showed that these treatments of DCs almost completely inhibited the cross-presentation of Hsp90–OVA (Fig. 9C). Taking into account the co-localization of exogenous Hsp90, OVA and proteasome subunit LMP-2 shown by the confocal laser study and the results from radicicol treatment, it was suggested that exogenous Hsp90 itself might act as a translocator of chaperoned OVA from early endoosme to cytosol for proteasomal degradation.

Hsp90–OVA complex is cross-presented by DCs in vivo

We examined whether our system worked *in vivo*. We immunized B6C3F1 mice with the Hsp90–OVA complex or OVA alone and evaluated the appearance of $CD11c^+$ DCs that could stimulate B3Z and KZO in the draining lymph nodes. Figure 10A and B show that $CD11c^+$ DCs from mice immunized with Hsp90–OVA elicited strong B3Z responses, whereas they did not cause KZO responses. In contrast, $CD11c^+$ DCs from mice immunized with OVA alone activated KZO, whereas stimulation of B3Z by them led to very weak responses compared with mice immunized with the Hsp90–OVA complex. These results were in agreement with previous findings shown in Fig. 4 that Hsp90–OVA was presented selectively and efficiently through the MHC class I pathway *in vitro*.

Hsp90–OVA complex elicits OVA-specific CTLs in vivo and protects mice from challenge with tumor cells expressing the OVA protein

We tested whether mice immunized with Hsp90–OVA developed OVA-specific CTL responses. C57BL/6 mice were immunized s.c. on days 0 and 7 with the Hsp90 (20 μg)–OVA (20 μg) complex in the absence of an adjuvant. As shown in Fig. 10C, immunization with Hsp90–OVA induced a strong and OVA-specific CTL response. Then, to study the efficacy of Hsp90–OVA to protect mice against grafted E.G7 tumor cells, mice (n = 10) were injected s.c. on days 0 and 7 with Hsp90 (20 μg)–OVA (20 μg), Hsp90 (20 μg), OVA (20 μg) or PBS, and 7 days after the second immunization, they were grafted i.d. with 10^5 E.G7 cells. All mice immunized with

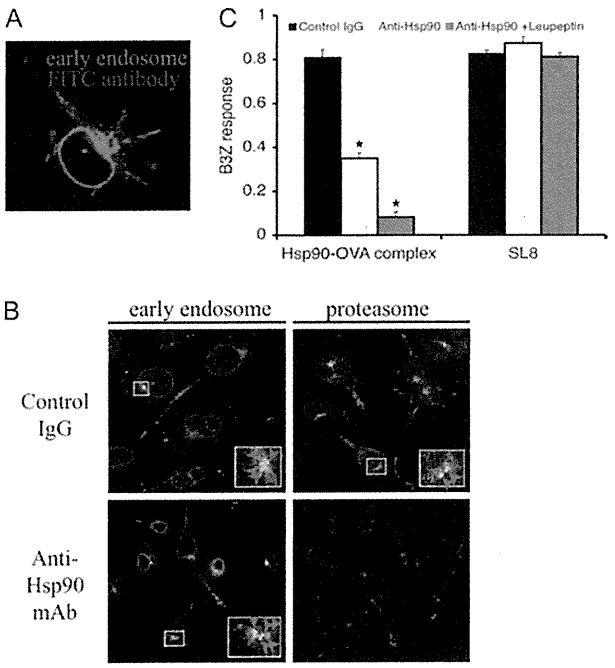

Fig. 9. Exogenous Hsp90 contributes to the translocation of chaperoned antigen from endosomes into cytosol. (A–C) One microgram of a FITC-antibody control protein, anti-Hsp90 mAb or rat IgG_{2a} control protein was mixed with the BioPORTER protein delivery reagent and added to each dish of DCs. After incubation for 4 h, the medium was removed and the cells were washed with RPMI twice to remove any antibodies that did not enter the cells. (A) Localization of transduced FITC-antibody control protein was examined. After incubation for 4 h, organelles were stained with an anti-Rab5 pAb for early endosomes conjugated with Alexa Fluor 594 and visualized with confocal laser microscopy (original magnification ×630). The BioPORTER/antibody complex localized in the cytosol instead of endosomes. (B) After the transduction of an anti-Hsp90 mAb or rat IgG_{2a} control protein, the cells were incubated with Hsp90–Alexa Fluor 594-labeled–OVA for 1 h, and then washed and fixed. Organelles were stained with an anti-Rab5 pAb for early endosomes and anti-LMP2 pAb for proteasomes, conjugated with Alexa Fluor 488 and visualized with confocal laser microscopy (original magnification ×630). Co-localization of internalized Hsp90–OVA and each organelle is indicated with arrows. Data are representative of three independent experiments. (C) After the transduction of an anti-Hsp90 mAb or rat IgG_{2a} control protein, the cells were incubated with leupeptin (200 μM) for 2 h, and then pulsed with Hsp90–OVA or SL8 for another 2 h, fixed, washed and cultured overnight with B3Z. The B3Z response was measured by the absorbance at 595 nm. Data are shown as means ± SD of three independent experiments. *, $P < 0.01$.

Hsp90, OVA or PBS developed tumors, whereas 60% of mice immunized with Hsp90–OVA remained tumor free (Fig. 10D). These results showed that the Hsp90-antigen peptide complex induced a strong CTL response to the chaperoned peptide and that this response was sufficiently strong to generate anti-tumor effects.

Discussion

Our results have revealed a novel mode of involvement of Hsp90 in antigen presentation by DCs: exogenous Hsp90 preferentially introduces the chaperoned antigen into the MHC class I pathway, resulting in efficient cross-presentation. Because there is a classical paradigm that extracellular antigens are presented by MHC class II molecules, it seemed significant to find that Hsp90 changed the fate of the associated antigen on antigen presentation. Recent reports demonstrated that Hsp70– and gp96–antigen complex facilitated antigen presentation in association with both MHC class I and class II molecules (13–15). Here, we examined whether an Hsp90–protein complex elicited $CD4^+$ T-cell responses through MHC class II presentation as well as $CD8^+$ T-cell responses and we found that the Hsp90–OVA complex induced preferential $CD8^+$ T-cell responses. Therefore, we further examined how Hsp90 directs the associated OVA to the cross-presentation pathway compared with the intracellular routing of OVA alone using laser confocal microscopy. We found that the Hsp90–OVA complex entered the MHC class I pathway, which is comprised of an endosomal pathway and proteasome–TAP-dependent pathway. In contrast, the soluble OVA was sorted into an endosome–lysosome pathway, which is regarded as a classical MHC class II pathway. Thus, the Hsp90–OVA complex and OVA followed different routes and it is very important to elucidate the role of Hsp90 in cross-presentation for the development of HSP-based cancer vaccines. We assumed that exogenous Hsp90 was taken up by receptor-dependent endocytosis. In fact, the Calderwood group has recently reported that the scavenger receptor expressed by endothelial cells-1 (SREC-1) acts as the Hsp90 receptor for cross-presentation expressed on DCs (16). Lakadamyali *et al.*(17) have demonstrated that early endosomes are comprised of two distinct populations: a dynamic population that matures rapidly toward the late endosome and lysosome, and a static early endosome that matures much more slowly. Interestingly, Burgdorf *et al.* (18) have demonstrated that a mannose receptor introduces exogenous OVA specifically into an $EEA\text{-}1^+$, $Rab5^+$-static early endosomal compartment for subsequent cross-presentation. In contrast, OVA endocytosed by a scavenger receptor did not co-localize with EEA1; instead, it co-localized with LAMP-1 in the lysosome as shown here, leading to presentation in the context of MHC class II molecules. Thus, we expect that Hsp90-specific receptors such as SREC-1 might introduce the Hsp90–OVA complex into the static early endosome for cross-presentation. Therefore, it should be examined whether the Hsp90–OVA complex is localized to the static early endosome after uptake through Hsp90 receptors, including SREC-1. More importantly, it should be clarified whether such Hsp90 receptors for cross-presentation bear the sorting motifs, which are responsible for introducing the Hsp90–OVA complex into the static early endosome. Identification and characterization of Hsp90 receptors have important implications for further understanding the biology of cross-presentation.

In our previous study, we observed that an exogenous Hsp90–'precursor peptide' complex localized to the early endosome but not the ER (12). Because our published data indicated that the Hsp90–peptide complex was cross-presented by a TAP-independent endosome-recycling pathway and this Hsp90-mediated cross-presentation was lactacystin-insensitive, we did not examine whether the Hsp90–precursor peptide (13mer) complex reached the proteasome. In cross-presentation of the Hsp90–OVA protein complex, we showed that part of the OVA chaperoned by

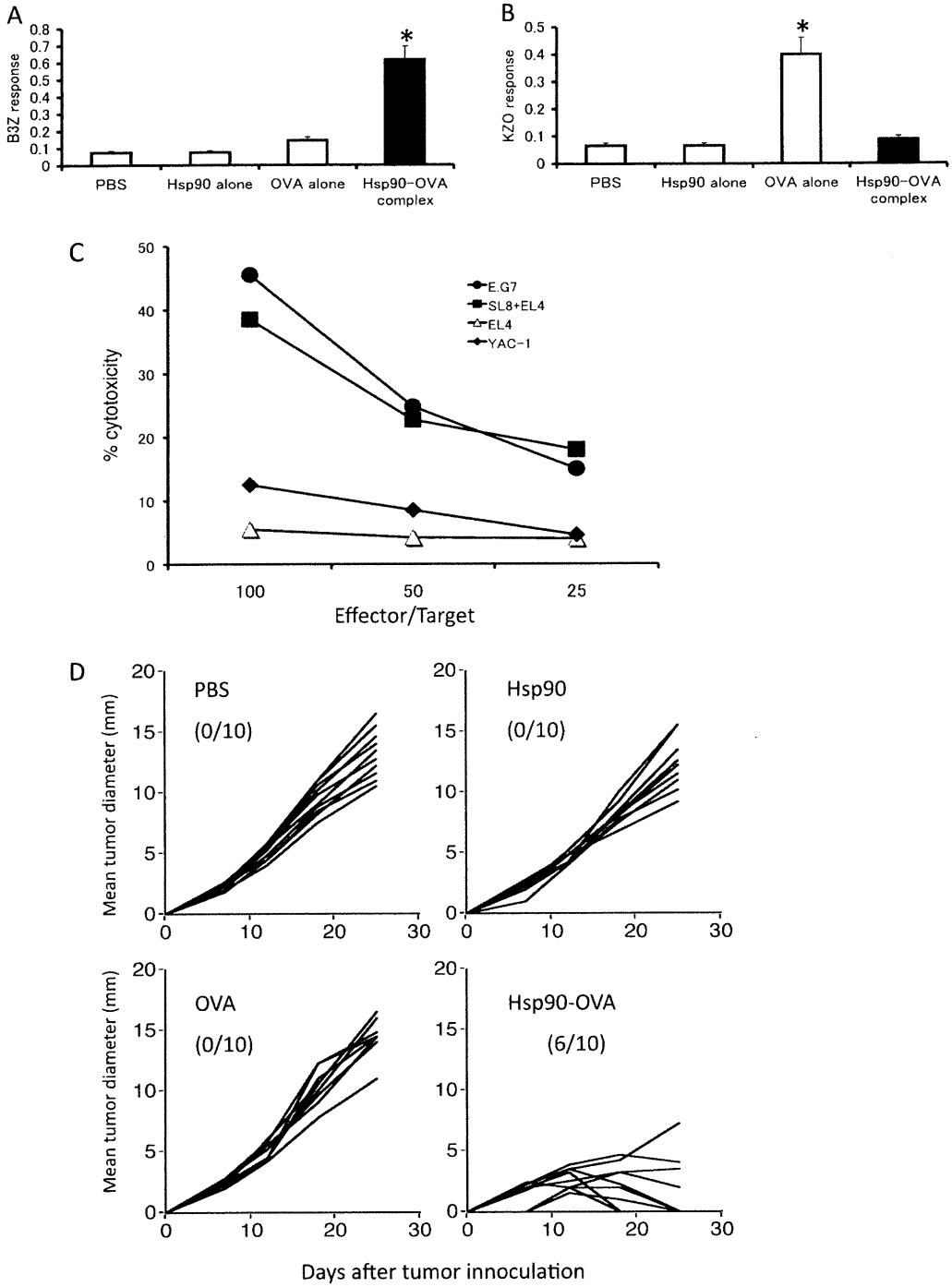

Fig. 10. Immunization with Hsp90–OVA complex elicits OVA-specific CTL responses *in vivo*. (A and B) B6C3F1 mice were immunized in their footpads with Hsp90 alone, OVA alone or a complex of them. Draining popliteal lymph nodes were removed after 4 h, and co-cultured overnight with B3Z (A) or KZO (B). The B3Z or KZO response was measured by the absorbance at 595 nm. Data are shown as means ± SD of three independent experiments. *, $P < 0.01$. (C) C57BL/6 mice were immunized s.c. with Hsp90–OVA, Hsp90 or OVA on days 0 and 7. Seven days after immunization, induction of SL8-specific CTL against E.G7 and EL-4 pulsed with SL8 peptide was measured by a standard 4-h chromium release assay. Cytotoxicity against E.G7 and EL4 loaded with SL8 (SL8 + EL4) showed statistically significant differences compared with EL4 or YAC-1 cells at all effector to target ratios ($P < 0.01$). Data are presented as means of triplicate samples and are representative of three independent experiments. (D) C57BL/6 mice were injected s.c. on days 0 and 7 with Hsp90–OVA, Hsp90, OVA or PBS. Seven days after the second immunization, mice were challenged i.d. with 1×10^5 E.G7 cells. Tumor size was measured in two dimensions twice a week. Then number of mice in which the tumor was rejected is indicated in parentheses. Data are representative of three independent experiments. Mean tumor diameter and survival on day 25 in the group of mice immunized with Hsp90–OVA showed the significant differences as compared with the other groups ($P < 0.001$ versus all other groups).

Hsp90 was transported out to cytosol for proteasomal degradation. These differences may reflect the need for proteasomal processing. More specifically, endosomal protease was sufficient to generate the SL8 epitope (8mer) from the precursor peptide, whereas OVA protein underwent both endosomal and proteasomal processing to generate the epitope. Further examination is required to elucidate the precise mechanism for the decision making on the destination of Hsp90-chaperoned antigens.

In addition, HSP–'peptide' complex-mediated cross-presentation has recently been well demonstrated by many groups, including ours. In contrast, there are only a few reports with regard to HSP–'protein' complex-mediated cross-presentation. Subjeck *et al.* have reported that an Hsp70 family member grp170–tyrosinase protein complex promotes cross-presentation and induces an anti-tumor effect. However, the intracellular route after uptake of the grp170–tyrosinase complex by DCs has not been explored. Thus, the precise mechanism, such as a translocator or a channel responsible for the translocation of the HSP–'antigen protein' complex from endosomes into cytosol for proteasomal degradation remains to be clarified. It has been suggested that, for TAP-dependent cross-presentation, exogenous antigen that translocates across the endosomal membrane to the cytosol is in a relatively unfolded state because membrane pores (~5–8 Å in a diameter) are not large enough for the passage of Hsp90 and OVA (>90 kDa with hydrodynamic radii >30 Å) (19, 20). Therefore, mildly acidic pH in the early endosome (pH 6.3) may be required to partially unfold the Hsp90–OVA complex to translocate it across the endosomal membrane and it may be renatured in the cytosol. Moreover, as the Hsp90–OVA complex is expected to be taken up via receptors such as SREC-1, the acidic condition might be necessary to dissociate the Hsp90–OVA complex from such receptors. In addition, Amigorena *et al.* have demonstrated that high pH (~7) in the phagosomes is required to prevent the excessive degradation of endocytosed antigen by lysosomal proteases for efficient cross-presentation. In line with these observations, we showed that the Hsp90–OVA complex was targeted to the early endosome, whose pH is believed to be relatively high (pH 6.5), leading to translocation of the Hsp90–OVA complex into cytosol without excessive degradation. However, our data using chloroquine suggested that mildly acidic pH (6.5) in the early endosome was required, probably because of the partial unfolding of the Hsp90–OVA complex as well as dissociation from Hsp90 receptors as described above. In our manuscript, we aimed at determining the underlying mechanism for this antigen translocation necessary for cross-presentation. We have observed that (i) the exogenous Hsp90–OVA complex co-localized with proteasome subunit LMP2 and (ii) blocking of interaction of the exogenous Hsp90–OVA complex and proteasomes by the introduction of an anti-Hsp90 antibody using BioPORTER resulted in the inhibition of cross-presentation. Although it was considered that the transduced anti-Hsp90 mAb also bound to endogenous Hsp90 as well as exogenous Hsp90, our data indicated that inhibition of the endogenous Hsp90 by radicicol did not affect the cross-presentation of the exogenous Hsp90–OVA complex. Furthermore, radicicol treatment did not affected the co-localization of the Hsp90–OVA complex and LMP2. Thus, exogenous Hsp90 seems to play an important role in cytoplasmic translocation of the endocytosed Hsp90–antigen complex. It has been shown that cytoplasmic Hsp90 is essential for the translocation of the diphteria toxin catalytic domain (21), *Clostridium botulinum* C2 toxin (22), fibroblast growth factor (FGF)-1 and FGF-2 (23). Thus certain toxins and growth factors depend on Hsp90 for efficient translocation from endosomes to cytosol. Very recently, Ichiyanagi *et al.* (20) have demonstrated that endogenous Hsp90 plays an essential role in cross-presentation of both exogenous OVA and cell-associated OVA by DCs. In agreement with their data, we showed that cross-presentation of soluble OVA was sensitive to the Hsp90 inhibitor radicicol, indicating that the endogenous Hsp90 might act as a translocator to the proteasome–TAP-dependent cross-presentation pathway for the exogenous antigen. These findings also suggest that endogenous Hsp90, as well as exogenous Hsp90, might help the exogenous Hsp90–OVA complex translocate into cytosol at the cytosolic face for cross-presentation. More importantly, it should be revealed how Hsp90–OVA complex escapes across the endosomal membrane. Experimental evidence supports existence of chaperones and co-chaperones such as Hsp90 and Hsc 73 on the endolysosomal membrane participating in lysosomal proteolytic pathways such as chaperone-mediated autophagy (24, 25). Moreover, Hsp90 forms multichaperone complex, which is consisted of Hsc73, p23 and Hop (26). Therefore, it is possible that these cytoplasmic chaperone complex might play an important role in this Hsp90-mediated cross-presentation and that endogenous Hsp90 might help to translocate the exogenous antigen from endosomal compartment to cytosolic proteasome for degradation. More importantly, it has been suggested that Sec61 complex played an important role in the translocation of exogenous antigens to cytosol for proteasomal degradation (27, 28). Therefore, it should be clarified whether the Hsp90–OVA complex is pumped out to cytosol through Sec61 complex. Furthermore, association of Hsp90 with the 20S proteasome has been shown to influence proteasomal enzymatic activity (29). Yamano *et al.* (30) have demonstrated that antigen processing by proteasomes is regulated by Hsp90 and PA28. They showed that up-regulation of Hsp90 and PA28 enhanced the antigen processing of OVA. Given our observation of the association of exogenous Hsp90 and the proteasome, exogenous Hsp90 might regulate proteasomal activity to create antigenic determinants. Together, our data show that TAP-dependent cross-presentation may require exogenous Hsp90 for the delivery of exogenous antigens into the cytosol for proteasomal proteolysis. The Hsp90 appears to represent an attractive candidate for vaccine development due to its ability to target DCs and to induce specific CTL without the need for an adjuvant.

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COMPARISON OF SPEEDY PCR-SSP METHOD AND SEROLOGICAL TYPING OF HLA-A24 FOR JAPANESE CANCER PATIENTS

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☐ *Human leukocyte antigen (HLA) typing is essential to carry out HLA-class I restricted antigenic peptide-based cancer immunotherapy. To establish a one-step polymerase chain reaction–sequence-specific primer (PCR-SSP) method, we designed two novel HLA-A24-specific primer sets and determined the optimal conditions for specific amplification. Then, we performed HLA-A24 typing of two healthy donors' and 17 cancer patients' peripheral blood with serological typing and PCR-SSP typing. Eleven of the 19 cases were determined HLA-A24-positive by the PCR-SSP method precisely; however, five cases showed false positive with serological analysis. Thus, for HLA-A24 typing in the Japanese population, the PCR-SSP method is faster and more accurate than serological typing.*

Keywords HLA-A24, HLA-typing, monoclonal antibody, PCR-SSP

INTRODUCTION

Human leukocyte antigen (HLA)-class I–restricted antigenic peptides are essential to establish cancer immunotherapy. Previously, we identified several HLA-A24–restricted antigenic peptides encoded within tumor-associated antigens (TAAs).[1–8] We have launched clinical trials of cancer vaccine therapy with HLA-A24–restricted antigenic peptides.[9,10] HLA-A24 typing is essential to carry out cancer vaccine therapy with HLA-A24–restricted peptides. There are several methods of HLA typing, and serological typing is the most common in routine analysis. However, recent

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advances in assigning HLA-class I alleles by techniques involving DNA analysis indicate that serological typing may not be sufficiently reliable.[11,12] One of the reasons is the limitations of HLA-A24–specific monoclonal antibodies (mAbs). Monoclonal antibodies, clone A11.1 M and C7709A2.6, are possible candidates for HLA-A24[13,14]; however, A11.1 M is also reactive for HLA-A11, which is also a common allele, and is not suitable for HLA-A24 detection. Thus, we evaluate clone C7709A2.6 for HLA-A24 detection.

In this report, to establish speedy and accurate HLA-A24 typing in Japanese, we compared serological typing with mAb C7709A2.6 and polymerase chain reaction (PCR)-sequence specific primer (SSP) with double HLA-A24-specific primer sets. mAb C7709A2.6 was positive for HLA-A24-positive cases' peripheral blood mononuclear cells (PBMCs). On the other hand, C7709A2.6 was partially false positive for HLA-A24-negative cases. On the other hand, the results of PCR-SSP analysis with two primer sets were completely identical with whole HLA-class I genotyping. These data suggest that the PCR-SSP method with two novel primers is a quick and accurate method for HLA-A24 screening.

EXPERIMENTAL

Flow Cytometric (FCM) Analysis and Monoclonal Antibodies (mAbs)

Peripheral blood mononuclear cells (PBMCs) were isolated by standard density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). The cells were incubated with a mouse anti-HLA-A24 monoclonal antibody (C7709A2.6, kind gift from Dr. P. G. Coulie) at saturating concentration for 30 min on ice, washed with phosphate-buffered saline (PBS), and stained with a polyclonal goat anti-mouse antibody coupled with FITC for 30 min. Samples were analyzed using a FACSCalibur analyzer (Becton Dickinson, Mountain View, CA, USA). An HLA-A24 positive EB virus-transformed B cell line, LG2-EBV (a kind gift from Dr. B. J. van den Eynde), was used as a positive control. HLA-class I negative leukemic cell line K562 was purchased from ATCC and used as a negative control.

One-Step PCR Analysis of Genomic DNA

Genomic DNA from 200 μL of peripheral blood was isolated with a QIAamp DNA mini kit (QIAGEN) as described in the manufacturer's protocol. Then, PCR amplification was performed in 20 μl of PCR mixture containing 1 μl of genomic DNA, 0.1 μl of Taq DNA polymerase (QIAGEN), and 12 pmol of primers. The PCR mixture was initially incubated at 94°C

for 2 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 68°C for 30 sec, and extension at 72°C for 30 sec. Primer pairs used for PCR analysis were as follows. The primer 1 set for HLA-A24-specific detection was composed of 5′-ACTGACCGAGAGAACCTGCGGAT-3′ and 5′-ACTTGCGCTTGGTGATCTGAGCC-3′ as sense and anti-sense with an expected PCR product size of 464 base pairs (bps). The primer 2 set for HLA-A24-specific detection consisted of 5′-ACAGACTGACCGAGAGAACC TGC-3′ and 5′-ACTTGCGCTTGGTGATCTGAGCC-3′ as sense and anti-sense with an expected PCR product size of 468 bps. As an internal control, an HLA common sequence was detected with the primer 3 set, 5′-ACGTGGACGACACGCAGTTCGTG-3′ and 5′-TTCCCGTTCTCCAGGTAT CTGCG-3′ as sense and anti-sense with an expected PCR product size of 713 bps. The PCR products were visualized with ethidium bromide staining under UV light after electrophoresis on 1.2% agarose gel. Nucleotide sequences of the PCR products were confirmed by direct sequencing using an ABI Genetic Analyzer PRISM 310 and an AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA, USA).

RESULTS

Novel HLA-A24–Specific Primer Sets and Determination of a Suitable Annealing Temperature for PCR-SSP

To establish the one-step PCR-SSP method, we designed two novel HLA-A24–specific primer sets 23 bps in length (Table 1 and Figure 1). To determine the appropriate annealing temperature, we performed PCR amplification with serial annealing temperatures to amplify the HLA-A*2402-positive and HLA-A*2402-negative genomic DNAs (Case #5 and #3). As shown in Figure 2, PCR bands were observed in both HLA-A*2402-positive and HLA-A*2402-negative samples with annealing temperatures of 60–66°C, suggesting that gene amplifications were not specific for HLA-A*2402. On the other hand, the PCR band could be observed in only the HLA-A*2402-positive sample with annealing temperatures of 68°C and

TABLE 1 PCR Primer Pairs

Primer	Orientation	Oligonucleotide Sequence (5′—3′)
Primer 1	sense	ACTGACCGAGAGAACCTGCGGAT
	antisense	ACTTGCGCTTGGTGATCTGAGCC
Primer 2	sense	ACAGACTGACCGAGAGAACCTGC
	antisense	ACTTGCGCTTGGTGATCTGAGCC
Primer 3	sense	ACGTGGACGACACGCAGTTCGTG
	antisense	TTCCCGTTCTCCAGGTATCTGCG

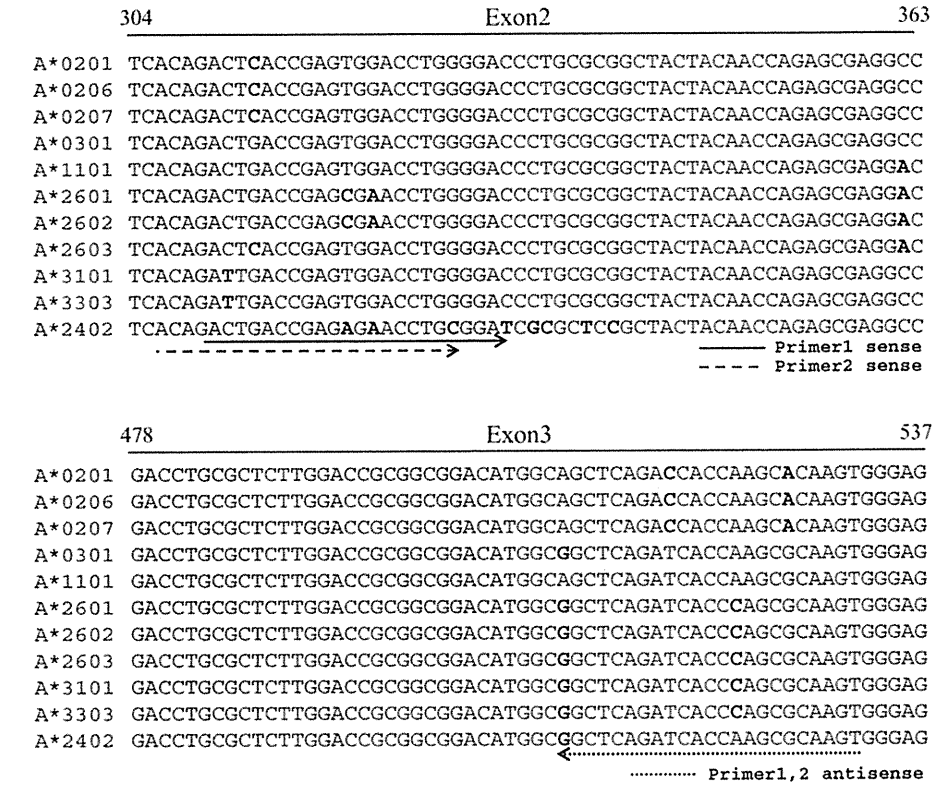

FIGURE 1 Gene specific primers for HLA-A*2402. Gene sequence alignments of HLA-class I molecules frequent in Japanese populations are shown. Bolds show the allele specific alignments. Numbers indicate numbers from the start codon. HLA-A24 gene specific primers were designed based on HLA-A24 specific alignments (primer 1 and primer 2).

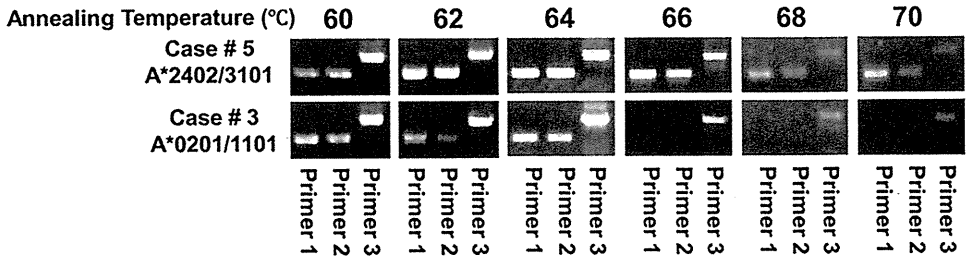

FIGURE 2 Determination of adequate annealing temperature for specific HLA-A24 detection. HLA-A24–positive and HLA-A24–negative genomic DNAs were amplified with HLA-A24-specific primer sets (primer 1 and primer 2). Primer 3 for a common HLA sequence was used as a positive control. Annealing temperatures were settled from 60°C to 70°C. Case #5 (HLA-A*2402/3101) and case #3 (HLA-A*0201/1101) genomic DNAs were used as positive and negative controls, respectively.

70°C. The PCR bands were confirmed to be identical with HLA-A*2402 sequence by DNA direct sequencing (data not shown). Thus, HLA-A24–specific amplification could be attained with annealing temperatures of 68°C and 70°C. Therefore, we have chosen 68°C as the annealing temperature for HLA-A24–specific amplification with these primer sets.

Detection of HLA-A24 with the PCR-SSP and Serological Methods

To compare the serological typing of HLA-A24 with the one-step PCR-SSP method, samples from two healthy donors and 17 cancer patients were evaluated. PBMCs from donors were isolated, and analyzed by FCM to detect the cell surface HLA-A24 molecules. The whole genomic DNA was isolated from whole blood and analyzed with one-step PCR-SSP method with 68°C for the annealing temperature. Figure 3 shows a representative HLA-A24-positive and -negative cases. HLA-A24 could be detected by both serological typing and PCR-SSP typing with HLA-A*2402-positive case (Case #8) specifically, suggesting that both PCR-SSP and serological typing work well.

Then, we compared several cases with PCR-SSP and FCM methods. Serologically HLA-A24-positive cases (Figure 4, upper panel) and serologically HLA-A24-negative cases (Figure 4, lower panel) were completely identical with the results of PCR-SSP analysis. On the other hand, some cases showed

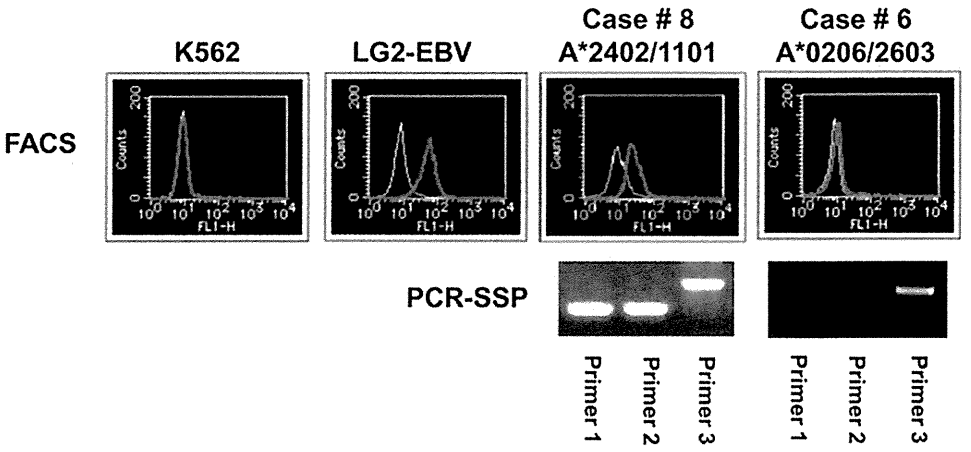

FIGURE 3 Specific detection of HLA-A24 with the serological method and PCR-SSP method. HLA-A24 molecule was detected with FCM analysis using mAb C7709A2.6 and PCR-SSP analysis using gene-specific primers. Cell surface HLA-A24 molecules were detected by flow cytometry. The PCR-SSP method was carried out with an annealing temperature of 68°C. K562 is an HLA-A24-negative cell line, and LG2-EBV is an HLA-A24-positive cell line. Case #8 HLA-A genotype was HLA-A*2402/1101 and case #6 was HLA-A*0206/2603.

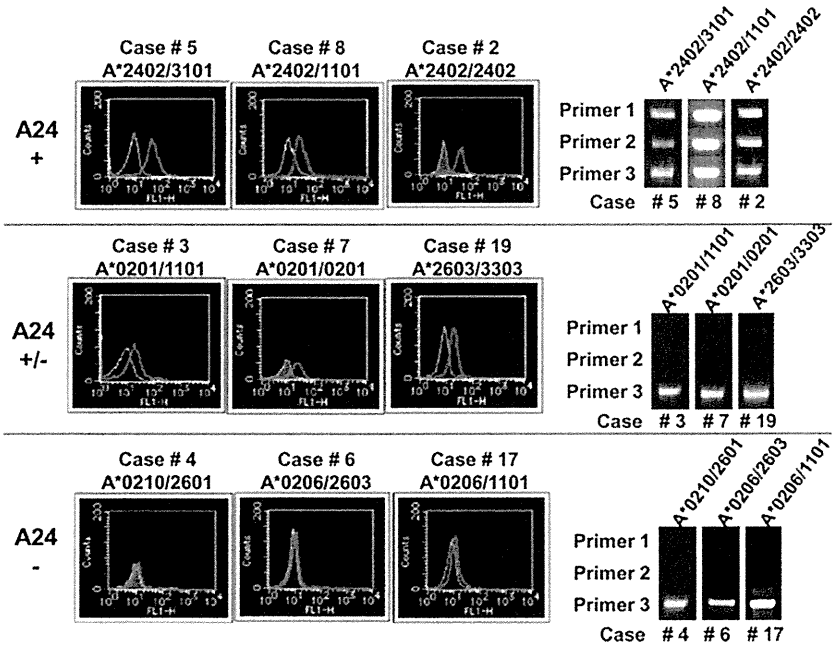

FIGURE 4 Serological HLA-A24–positive, weakly positive, and negative cases. Representative serological HLA-A24–positive, weakly positive, and negative cases are shown (upper, middle, and lower panel). PCR-SSP analysis data are shown in the right column. Corresponded HLA-A genotypes are shown.

TABLE 2 Summary of Flow Cytometry and PCR-SSP Analysis of HLA-A24

Case	Sex	Donor Type	Flow Cytometry	PCR-SSP	Genotype (HLA-A)
1	Female	Healthy donor	+	+	**2402**/3303
2	Male	Healthy donor	+	+	**2402/2402**
3	Male	Lung cancer	+/−	−	0201/1101
4	Male	Colon cancer	−	−	0210/2601
5	Female	Lung cancer	+	+	**2402**/3101
6	Male	Lung cancer	−	−	0206/2603
7	Female	Breast cancer	+/−	−	0201/0201
8	Male	Lung cancer	+	+	**2402**/1101
9	Male	Lung cancer	+	+	**2402**/0207
10	Female	Breast cancer	+	+	**2402**/0206
11	Female	Breast cancer	+	+	**2402/2402**
12	Male	Lung cancer	n.d.	+	**2402**/0206
13	Male	Lung cancer	n.d.	+	**2402**/3303
14	Male	Lung cancer	+/−	−	0207/1101
15	Female	Breast cancer	+/−	+	**2402/2402**
16	Male	Colon cancer	+/−	−	**2402/2402**
17	Male	Lung cancer	−	−	0206/1101
18	Female	Head and neck cancer	+/−	−	0201/0201
19	Male	Lung cancer	+/−	−	2603/3303

n.d.: not detected.

serological HLA-A24–weakly positive as compared with serologically HLA-A24–positive cases with mAb C7709A2.6 (Figure 4, middle panel). Part of these serological weakly positive cases were proved to be HLA-A24-negative (false positive) by one-step PCR-SSP analysis. The whole HLA-class I typing was completely consistent with the results obtained using the one-step PCR-SSP method. Five of the 19 samples were proved to be serological false positives (Table 2). With one-step PCR-SSP analysis, all 19 cases were analyzed appropriately. These data suggested that the one-step PCR-SSP method was suitable for routine HLA-A24 typing in the Japanese population.

DISCUSSION

Recently, a large number of cancer-related antigenic peptides, including HLA-A24–restricted peptides, has been reported, and cancer immunotherapy with such antigenic peptides has been initiated all over the world.[15] Some cases were reported to show clinical responses, including tumor regression and decrease in markers. These data will accelerate the use of cancer immunotherapy as a new treatment modality. Because HLA binding affinities of antigenic peptides depend on HLA-types, HLA-typing is essential to carry out cancer immunotherapy with HLA-restricted antigenic peptides. The PCR-SSP method and serological typing are candidates to detect specific HLA alleles. Several reports have shown that there were discrepancies between DNA typing and serological typing.[11,12] There are already several reports of methods for DNA typing of specific alleles.[16–19] However, there is no report about the typing of HLA-A24, which is the most frequent allele in Japanese and Asian populations and is also frequent in other ethnicities. Thus, the PCR-SSP method for HLA-A24 is required.

In this article, we report the establishment of a PCR-SSP method with novel HLA-A24-specific primers within exon 2 and exon3. Exon2 and exon 3 encode the alpha-1 and alpha-2 domains of the HLA molecule, which then bind to antigenic peptides and are highly polymorphic domains. Thus, exon 2 and exon 3 are suitable to design primers for PCR-SSP. As shown in Figure 1, there are a couple of additional HLA-A*2402 specific sequences in exon 2, which are potentially suitable for design of sense primers. Actually, prior to this study, we evaluated two more HLA-A*2402 specific primers (5′-AGAGAACCTGCGGATCGCGCTCC-3′ and 5′-GACCGA GAGAACCTGCGGATCGC-3′) as sense primers; however both of those primers did not show specific amplification, even with 70°C for annealing. Thus, we decided to use two primers (primer 1 and primer 2), and we think these two primers are optimized for specific detection. This method is

speedy, taking only 2 h to carry out. Furthermore, this PCR-SSP method has high specificity compared with serological typing with mAb C7709A2.6. This suggests that PCR-SSP analysis is more suitable for daily screening of HLA-A24. However, since the PCR-SSP method detects only several HLA-A24–specific DNA sequences, it cannot assure the presence of functional HLA-A24 molecules on the cell surface. Ishikawa et al. showed that point mutations within the HLA-A2 coding region caused a stop codon, which resulted in immature and nonfunctional HLA molecules.[20] Laforet et al. showed that an intronic mutation caused lower expression of the HLA-A24 allele.[21] Thus, we might not be able to detect such variants with the PCR-SSP method, and the functional cell surface expression of the HLA-A24 molecule is essential to carry out antigenic peptide–based cancer immunotherapy. Therefore, it might be important to perform FCM analysis post–PCR-SSP screening to assure the presence of functional HLA-A24 molecules.

CONCLUSION

We established a one-step PCR-SSP method to detect HLA-A*2402 in the Japanese population. This method can be completed in about two hours, and is not expensive per test; thus it is suitable for daily screening for HLA-A24.

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Phase I clinical trial of survivin-derived peptide vaccine therapy for patients with advanced or recurrent oral cancer

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Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is abundantly expressed in most malignancies, but is hardly detectable in normal adult tissues. Previously we have identified a human leukocyte antigen (HLA)-A24-restricted antigenic peptide, survivin-2B80-88 (AYACNTSTL), recognized by CD8$^+$ cytotoxic T lymphocytes (CTL). Survivin-2B80-88-specific CTL were induced efficiently from peripheral blood mononuclear cells (PBMC) of oral cancer patients after stimulation with the peptide *in vitro*. We conducted a phase I clinical study to evaluate the safety and the efficacy of survivin-2B80-88 peptide vaccination in HLA-A24-positive patients with advanced or recurrent oral cancer. The vaccines were given subcutaneously or intratumorally six times at 14-day intervals. Eleven patients were enrolled and 10 patients completed the vaccination protocol. No adverse events were observed in any patients. In two patients, the levels of serum squamous cell carcinoma (SCC) antigen decreased transiently during the period of vaccination. Tumor regression that was compatible with a partial response (PR) was noted in one patient. The remaining nine patients experienced progressive disease (PD). Immunologically, an increase of the peptide-specific CTL frequency was detected in six of the eight patients evaluated by HLA-A24/peptide tetramer analysis. The present clinical trial revealed that survivin-2B peptide vaccination was safe and had therapeutic potential for oral cancer patients. However, subsequent clinical trials in combination with various adjuvant drugs will be required to improve the immunological and therapeutic efficacy. This trial was registered with University Hospital Medical Information Network (UMIN) number UMIN000000976. (*Cancer Sci* 2011; 102: 324–329)

Oral cancer consistently ranks as one of the 10 most frequently diagnosed cancers worldwide.[1] It encompasses a range of malignant tumors arising from various diverse and complex structures that have major physiological and aesthetic importance. For most early stage oral cancers, high cure rates are achieved with either surgery or definitive irradiation and both speech and swallowing functions can often be preserved. On the other hand, locally advanced or recurrent oral cancers are usually treated with combination therapy consisting of either surgery followed by postoperative chemoradiation or chemoradiation with surgical salvage if needed. However, most patients remain at high risk for locoregional recurrence and distant metastasis.[2] Therefore, advances in new therapeutic modalities such as tumor-specific immunotherapy for patients with locally advanced or recurrent oral cancers are urgently needed.

A large number of tumor-associated antigens have been identified from melanomas and other cancers, and clinical trials of peptide-based immunotherapy have been carried out. Melanoma antigen peptides were the first to be tested in phase I and phase II studies for active immunization of metastatic melanoma patients.[3,4] During the first stage of the studies, clinical responses were observed in Europe and the United States.[5,6] However, in 2003, Rosenberg *et al.*[7] reported that <5% of patients who received peptide vaccines such as gp100, MART-1 and tyrosinase plus IL-2 showed an overall objective response (complete response [CR] + partial response [PR]). On the other hand, investigational immunotherapy that targeted MAGE-A3 tended to reduce the risk of recurrence by 27% when used as an adjuvant therapy with surgery in stage IB/II non-small-cell lung cancer. Furthermore, enrolment in the global phase III trial of adjuvant MAGE-A3 for non-small-cell lung cancer has already started according to a certain European Union (EU)-based pharmaceutical company. This finding provides hope for current and future immunotherapies and has accelerated a variety of investigations concerned with human tumor immunology.

Survivin is a recently characterized inhibitor of apoptosis protein (IAP) that is abundantly expressed in most solid and hematological malignancies, but is barely detectable in normal adult tissues.[8] It has been shown to increase tumor resistance to apoptotic stimuli such as radiation and chemotherapy.[9,10] A number of reports have demonstrated that survivin expression in cancer cells has a prognostic value and is associated with increased tumor recurrence and a lower survival rate,[11–16] although the opposite correlation is observed in certain cancers.[17] We previously reported that survivin-2B, a splicing variant of survivin, is also expressed abundantly in various tumor cell lines and the survivin-2B80-88 (AYACNTSTL) peptide derived from the exon 2B-encoded region is recognized by CD8$^+$ cytotoxic T lymphocytes (CTL) in the context of human leukocyte antigen (HLA)-A24 molecules.[18] The CTL specific for this peptide were successfully induced from PBMC in six of seven HLA-A24-positive patients (83%) with colorectal cancers and exerted cytotoxicity against HLA-A24-positive/survivin-positive adenocarcinoma cells.[19] Furthermore, we recently demonstrated that survivin-2B peptide-specific CTL were induced in four of eight (50%) HLA-A24-positive patients with oral cancer with over stage II progression.[20] Based on these observations, a phase I clinical study of survivin-2B peptide vaccination was initiated for patients with locally advanced or recurrent oral cancer. The present clinical trial demonstrated the safety and suggested the marginal clinical effectiveness of the survivin-2B peptide vaccination alone for oral cancer patients.

Materials and Methods

Eligibility criteria. The study protocol was approved by the Clinical Institutional Ethical Review Board of the Medical

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