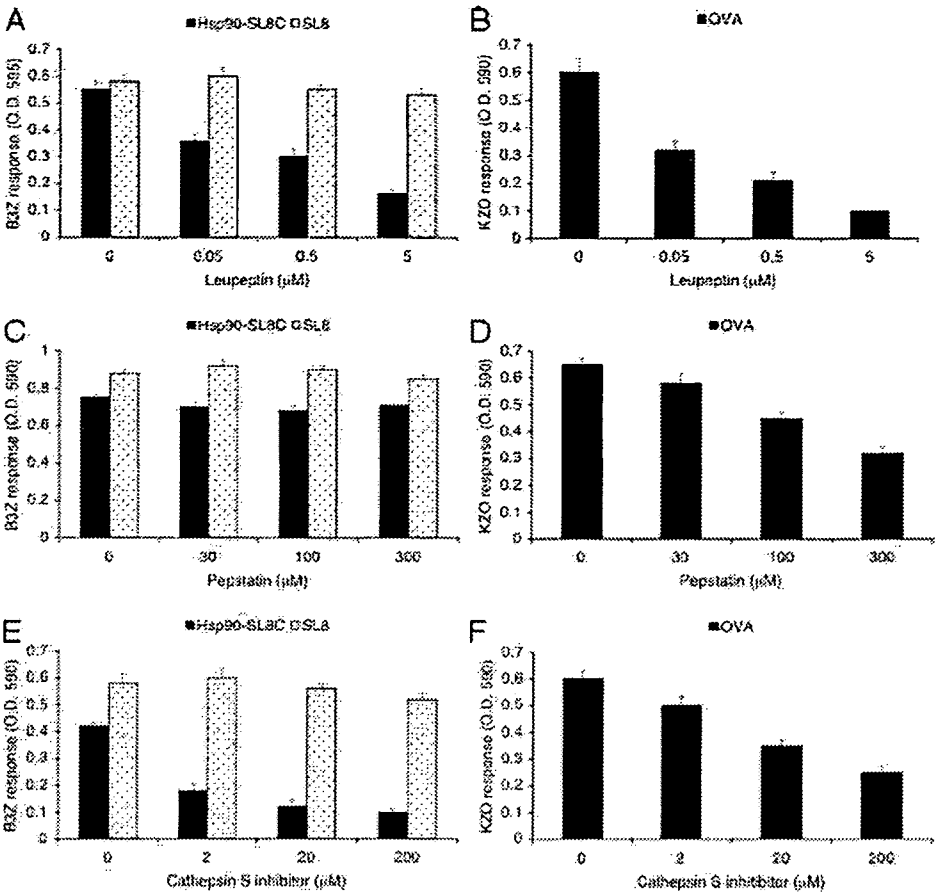

FIGURE 10. The Hsp90-chaperoned precursor peptide is processed by cysteine/serine proteases in the endosomes. *A–F*, DCs were preincubated with (*A* and *B*) leupeptin, (*C* and *D*) pepstatin, or (*E* and *F*) a cathepsin S inhibitor at 37°C for 2 h, then pulsed with Hsp90-SL8C complexes, SL8 peptide, or OVA protein for 2 h. The DCs were then fixed, washed, and cultured overnight with B3Z or KZO cells. The β-galactosidase activity was measured at the absorbance at 595 nm.

were generated from the Hsp90-precursor peptide complex through the endosomal pathway.

Hsp90-chaperoned peptides are transferred to recycling MHC class I molecules in early endosomes

Recycling of endocytosed MHC class I molecules back to the cell surface has been observed (32). Some of the recycling MHC class I molecules can be loaded into early endosomes with peptides derived from endocytosed molecules. Therefore, to confirm whether this presentation really used the recycling MHC class I molecules, we treated BMDCs with primaquine, which blocks the membrane recycling pathway. BMDCs incubated in the presence of this drug could not present the Hsp90-chaperoned SL8C (13 mer)-derived SL8 peptide (Fig. 9*D*). This result indicated that Hsp90-chaperoned precursor peptides or processed peptides could enter into recycling endosomes and be transferred onto recycling MHC class I molecules, which went back to the cell surface, resulting in the stimulation of B3Z T cell hybridoma. Furthermore, to analyze the involvement of vacuolar acidification of endosomal compartments, BMDCs were incubated with Hsp90-SL8C precursor peptide complexes in the presence of chloroquine, a known inhibitor of acidification of endosomal compartments. Chloroquine treatment resulted in strong inhibition of the Hsp90-mediated presentation, without affecting SL8 peptide presentation, showing that acidification of endosomal compartments was necessary for Hsp90-chaperoned precursor peptide processing (Fig. 9*E*).

Hsp90-chaperoned peptides are processed by endosomal protease

We used protease inhibitors to investigate how proteolytic processes were involved in this Hsp90-mediated TAP-independent cross-presentation pathway. We found that, in wild-type BMDC, a broadly active cysteine protease inhibitor, leupeptin, almost completely inhibited the cross-presentation of Hsp90-SL8C precursor peptide complexes (Fig. 10*A*). In contrast, the aspartic protease inhibitor pepstatin did not affect the cross-presentation (Fig. 10*C*). The concentration of leupeptin or pepstatin used was sufficient to inhibit cysteine proteases or aspartic protease because it completely blocked the presentation of soluble OVA on MHC class II molecules detected by I-A^k-specific $CD4^+$ T cell hybridoma KZO (Fig. 10, *B* and *D*). These results indicated that cysteine proteases are required for the Hsp90-mediated cross-presentation.

We next studied the role of cathepsins in the Hsp90-mediated vacuolar cross-presentation. Cathepsins S, B, and L are known to be the major cysteine proteases in endocytic compartments. We therefore examined the roles of various cathepsins in this pathway. A cathepsin B- or cathepsin L-specific inhibitor did not affect Hsp90-mediated cross-presentation (data not shown), whereas a cathepsin S inhibitor clearly blocked cross-presentation (Fig. 10*E*), as well as the presentation of soluble OVA on MHC class II molecules detected by KZO T cell hybridoma (Fig. 10*F*). Cathepsin S is preferentially expressed in APCs, including DCs, macrophages, and B cells within endocytic compartments. Therefore, our data indicated that cathepsin S was a critical enzyme in TAP-independent Hsp90-mediated cross-presentation to MHC class I molecules and that antigenic precursor peptides were indeed processed to epitope peptides, followed by association with MHC class I molecules in endosomal compartments.

Hsp90-protein Ag complex is cross-presented by BMDCs

Lastly, we evaluated cross-presentation of the in vitro-generated Hsp90-OVA protein complex. In vitro generation of Hsp90-OVA

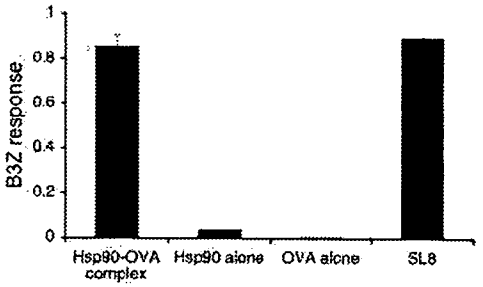

FIGURE 11. The Hsp90-OVA complex is presented by BMDCs through the MHC class I pathway. BMDCs were pulsed with Hsp90 alone, OVA alone, a complex of them or SL8 for 2 h at 37°C, then fixed, washed, and cultured overnight with B3Z. The β-galactosidase activity was measured at the absorbance at 595 nm.

complexes was performed and confirmed according to the method described in *Materials and Methods.* BMDCs were pulsed with Hsp90 alone, free OVA, or a complex of them generated in vitro for 2 h at 37°C, then fixed, washed, and cultured with B3Z $CD8^+$ T cell hybridoma. Hsp90-OVA elicited strong B3Z responses, while Hsp90 or OVA alone did not induce a B3Z response (Fig. 11). Thus, Hsp90-chaperoned protein Ag as well as peptide is efficiently cross-presented by BMDCs.

Discussion

We have shown here that Hsp90-peptide complexes could induce strong CTL responses, leading to efficient antitumor immunity via cross-presentation pathway. Interestingly, Hsp90-mediated cross-presentation is independent of TAP and sensitive to chloroquine, suggesting that processing and loading of peptides onto MHC class I occurs via the endosomal pathway. Although Binder et al. (33) have demonstrated that exogenous Hsp70 and gp96-mediated cross-priming is dependent on the TAP system in the peritoneal macrophage and macrophage cell line RAW264.7, we used immature BMDCs for Ag-presentation assay. In addition, they used *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) for introduction of HSP-peptide complexes into the cytosol of the macrophages. By contrast, we used exogenous Hsp90-peptide complex-pulsed BMDCs for the detection of the in vitro cross-presentation so as to mimic the situation that HSP-peptide complexes would be released into the extracellular milieu as a consequence of pathological cell death. In fact, Schoenberger et al. (34) demonstrated that cells deficient in TAP were still able to cross-present as efficiently as wild-type cell.

We have shown that exogenously loaded Hsp90 trafficked to early endosomes via receptor-mediated endocytosis and colocalized with recycling MHC class I molecules in the early endosomes where the exchange of the Hsp90-chaperoned-peptides might occur. Recent reports have identified several pathways wherein peptides exchange onto recycling MHC class I molecules occurs within early endosomal compartments (32, 35). Such trafficking pathways for recycling MHC class I molecules bear broad similarities to that observed for Hsp90. Therefore, we propose that, for the Hsp90-chaperoned peptide, the early endosome is a site for peptide exchange onto class I molecules for subsequent presentation. In addition, we have shown that the cysteine protease cathepsin S plays an important role in the generation of MHC class I peptides in endosomes. Rock and colleagues (36) have demonstrated that cathepsin S is a key enzyme for the generation of exogenous OVA-derived SL8 peptide, which is presented by a TAP-independent pathway. These facts indicate that endosomal cathepsin S might be necessary for the generation of Ag peptides, cross-presented by DCs. Further research defining the precise mechanisms of peptide exchange and processing may reveal a new paradigm for cross-presentation. Nicchitta and colleagues (5) have shown that gp96 internalizes by receptor-mediated endocytosis trafficked to an FcR and MHC class I-positive endocytic compartment and does not access the ER of BMDCs. These observations are consistent with our data. Taken together, it is suggested that BMDCs bear cell-surface receptors that are capable of directing HSP-peptide complexes into the class I Ag-presentation pathway. We are currently investigating the Hsp90-specific receptor on the APCs, which is responsible for the cross-presentation.

In contrast, immunization with the Hsp70-peptide complex elicited only weak CTL responses even though an Hsp70-antigenic peptide complex could be generated. The mechanistic details causing drastic differences between Hsp90 and Hsp70 in CTL induction remain to be determined.

These results indicate that Hsp90 serves as a powerful danger signal and elicits prompt protective immune responses against infection and cellular stress. Although, compared with the TAP-dependent pathway, the TAP-independent pathway is less effective under stress conditions, very rapid generation of protective immune responses could be beneficial against life-threatening events.

We have also demonstrated that Hsp90-chaperoned Ags cross-presented by BMDCs elicit strong Ag-specific CTL induction in vivo and an antitumor therapeutic effect. Although we used human tumor Ag survivin-2B as a surrogate Ag in the HLA-A*2402 transgenic mouse system, leading our tumor model highly immunogenic, treatment with Hsp90-survivin-$2B_{80-88}$ complexes showed significant therapeutic effect compared with treatment with survivin-$2B_{80-88}$ emulsified in IFA. The results suggested that Hsp90 might be a promising candidate for a well-tolerated adjuvant. Taken together, these results suggest new avenues for Hsp90-based immunotherapy in viral infection as well as anticancer vaccination.

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Disclosures

The authors have no financial conflict of interest.

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