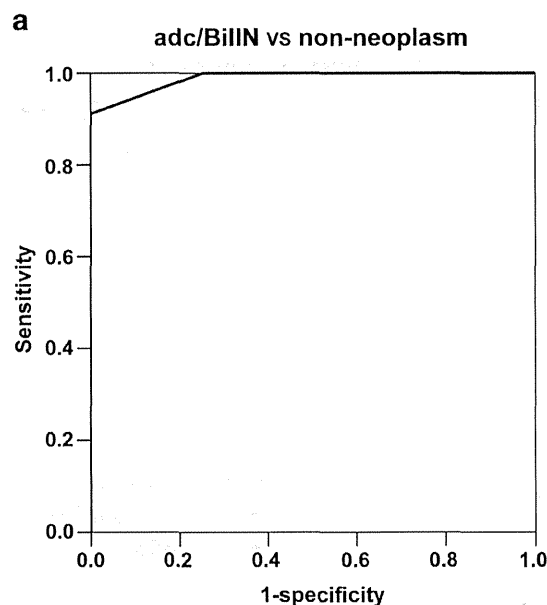


Fig. 2 ROC curve analysis of cldn18, maspin, and p53 in surgical specimens. **a** ROC curves were calculated according to multiplication scores that represented the degrees of immunoreactivity with a scale of 0 to 30 for each antibody as described in “Materials and methods.” Immunoreactivity against maspin was separately evaluated in the cytoplasm (C) and nucleus (N). Cutoff values were calculated from the ROC curves to distinguish adc/BilIN from non-neoplastic epithelium (*non-ne*). **b** Accuracy was measured by the AUC. The 95 % CIs for AUC and best cutoff values with the highest accuracy are summarized in the tables. **c** *Dot plots* show the distribution of multiplication scores for each antibody. In each panel, the *bold horizontal line* represents the best cutoff value to divide the cases into two groups, adc/BilIN (*above the line*) and non-ne (*below the line*). *CI* confidence interval

positive for maspin (5 of 22 non-neoplastic cases). However, we classified these as negative for maspin because H&E staining clearly demonstrated them as non-neoplastic (data not shown). To distinguish neoplasms from non-neoplastic epithelia, we analyzed the combined scores from the binary values for cldn18, maspin (N), and p53, as described for the surgical specimens. The AUC was 0.990 [95 % CI, 0.964–100] (data not shown). As shown in Figs. 4b, c, and d, at a cutoff value of 1, all 18 specimens with adenocarcinoma/BilIN and 4 of 19 with non-neoplastic epithelium were distinguished as neoplastic. The sensitivity and specificity were 100 and 78.9 %, respectively. At a cutoff value of 2, all 18 specimens with adenocarcinoma/BilIN and 1 of 19 with non-neoplastic epithelium were distinguished as neoplastic. The sensitivity and specificity were 100 and 94.7 %. When the cutoff value was set at 3, 11 of 18 specimens with adenocarcinoma/BilIN and none of those with non-neoplastic epithelium were distinguished as neoplastic. The sensitivity and specificity were 61.1 and 100 %, respectively. A cutoff value of 2 had the highest sensitivity and specificity, and the score provided good separation of neoplasm from malignancy-undetermined atypical epithelium (18/21, 85.7 %) in cases that showed nuclear atypia but could not be proven as adenocarcinoma with a biopsy. We examined seven percutaneous liver biopsy specimens and found that a cutoff value of 2 had the highest sensitivity and specificity (Supplementary Fig. S4).

Double staining of cldn18 and maspin

We examined 14 surgical specimens of biliary tract cancer by double staining for cldn18 and maspin. The staining pattern was coincident with those expected from the single staining patterns of cldn18 and maspin (Fig. 4e). Some specimens of neoplastic epithelium were immunoreactive for both cldn18 and maspin (12/14, 86 %); others were reactive only for cldn18 (2/14, 14 %), indicating that all examined biliary tract adenocarcinomas were positive for at least cldn18 or maspin. Among the 14 specimens, 9 had BilINs and all were positive for at least one marker. All of the specimens with BilIN-1 (3/3) and BilIN-3 (3/3) and 1/3 of those with BilIN-2 were double-



AUC	95%CI	
	Lower	Upper
0.989	0.978	0.999

Score (binary value)	Sensitivity		Specificity	
	≥1	≥2	≥1	≥2
	1.000	0.911	0.746	1.000
	0.511		1.000	

Score (binary value)	adc/BilIN		non-neoplasm	
	0	1	0	1
0	0	48	0	15
1	10	0	0	0
2	40	0	0	0
3	41	0	0	0
Total	91	63		

Fig. 3 ROC curve analysis in surgical specimens performed by using combined parameters of cldn18, maspin, and p53. **a** The ROC curve was calculated according to the combined binary values of the multiplication scores for each antibody. Briefly, each of the multiplication scores was converted to a binary value (0 for immuno-negative and 1 for immuno-positive) on the basis of the best cutoff values for each antibody (refer to Fig. 2b). Then, the binary values were combined, to give scores of 0: all immuno-negative to 3: all immuno-positive. Cutoff values were calculated from the ROC curve to distinguish adc/BilIN from non-neoplasm. Accuracy was measured by AUC. **b** Sensitivity and specificity for each cutoff value are summarized. With a cutoff value of 2, where any two antibodies produced immuno-positive staining, 81/91 adc/BilIN and none of non-neoplasm were evaluated as neoplastic with the highest sensitivity and specificity

positive, and 2/3 of those with BilIN-2 were positive only for cldn18. In contrast, most non-neoplastic specimens were negative for both cldn18 and maspin, except for one that was positive for maspin (1/14, 7%).

Discussion

The aim of this study is to provide a new approach to distinguish biliary tract carcinoma and BilIN from non-neoplastic epithelia with high sensitivity and specificity. We achieved this by a combination of immunohistochemical staining for cldn18, maspin, and p53. Expression of these three antigens has been reported in association with biliary tract carcinomas [20, 25, 32, 34–36], but immunostaining of each individual marker seems to be insufficient to distinguish biliary tract carcinoma/BilIN from non-neoplastic epithelia with certainty. For example, some diagnoses remained controversial if staining for cldn18 or maspin was weakly positive or if cldn18 was detected only in the cytoplasm, even though biliary tract carcinoma/BilIN is usually thought to overexpress cldn18 and maspin [20, 32, 34]. In addition, the prevalence of p53 expression in biliary tract carcinoma/BilIN is relatively low (approximately 30 % at most) with heterogeneous staining patterns even in a single specimen, although the p53 nuclear staining pattern is conspicuous and easy to evaluate in adenocarcinoma. Considering these advantages and disadvantages, we used a panel of all three immunohistochemical markers to reliably detect biliary tract carcinoma/BilIN.

First, we examined surgical specimens of biliary tract cancer by immunohistochemical staining for each marker, independently. Staining with each of the three antibodies seemed to demonstrate lack of homogeneity within an individual adenocarcinoma. In particular, almost every adenocarcinoma in the present study showed variable intensity and proportion of staining for cldn18 and maspin, not in association with histological differences. This may be attributed to genetic heterogeneity as neoplasms are genetically heterogeneous (interindividually and intraindividually), which is closely related to their progression and treatment response [34].

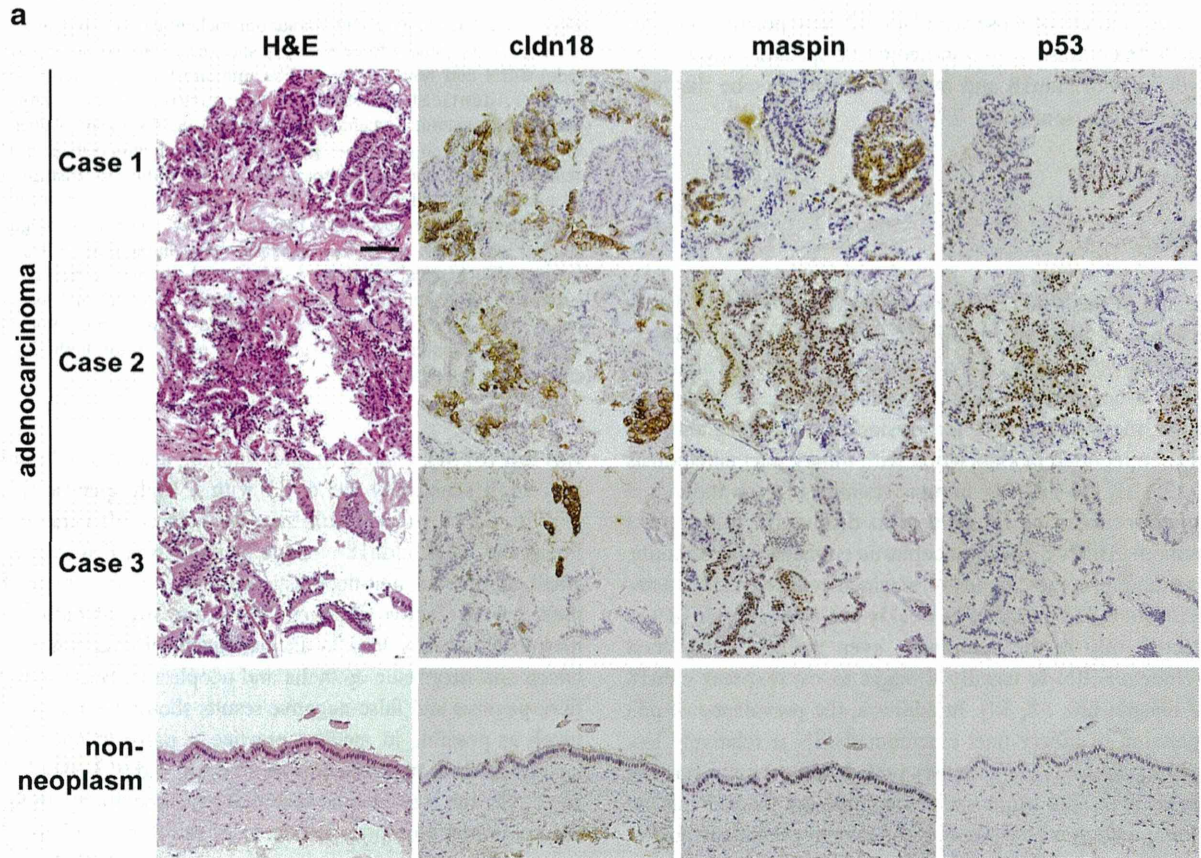
In the present study, we defined a parameter to maximize the reproducibility and accuracy of the immunohistochemical evaluation. This parameter, designated as the multiplication score, was calculated by multiplying the intensity (4 grades) and proportion (11 grades) of immunoreactivities for each antibody. All ROC curves revealed that sensitivity and specificity of the three markers are high enough to distinguish biliary tract adenocarcinomas/BilIN from non-neoplastic epithelia. Individually, maspin (N) immunostaining showed relatively high sensitivity (85.6 %) and specificity (77.8 %) in detecting adenocarcinoma and BilIN. p53 immunostaining showed the highest specificity (100 %), but the sensitivity

Fig. 4 **a** H&E staining and immunohistochemical staining in three presurgical endobiliary forceps biopsy specimens with adenocarcinoma (adc)/BilIN and non-neoplasm. The immunostaining patterns were almost identical to those in the surgical specimens. **b** Immunohistochemical staining results based on the binary values to give scores of 0: all immuno-negative to 3: all immuno-positive. **c** The sensitivity and specificity for each cutoff value to distinguish adenocarcinoma (adc)/BilIN from non-neoplasm are summarized. **d** The sensitivity and specificity for each cutoff value to distinguish atypical epithelium from non-neoplasm are summarized. **e** Double immunohistochemical staining of cldn18 and maspin in the surgical specimens. The staining patterns of cldn18 (brown) and maspin (blue) were coincident with those expected from staining for each individually. A non-neoplastic gland was negative for both cldn18 and maspin (asterisk)

was low (61 %). Cldn18 immunostaining also showed markedly high sensitivity (95.6 %) with a high specificity of 96.8 %, which would result from the high cutoff value of 6. Focal and weak cldn18-positive staining was detected in a small number of non-neoplastic epithelia, especially in the presence of reactive changes. Therefore, use of cldn18 or maspin alone may lead to an inadequate differentiation between non-neoplastic epithelia and neoplasms. Because both false-positive and false-negative results should be avoided as much as possible in medical practice, a panel of antibodies against all three markers, cldn18, maspin, and p53, should be used to increase diagnostic sensitivity and specificity. In fact, the combined multiplication score of the three markers improved the accuracy, as compared to the three individual scores (Fig. 2 and Supplementary Fig. S3B).

The combined multiplication score successfully distinguished neoplasms from non-neoplastic epithelia in the surgical specimens of the biliary tract; however, the scores were complicated to calculate and inconvenient for clinical application. Therefore, before combining the three parameters for cldn18, maspin (N), and p53, we converted each of the multiplication scores to binary values (0 for immuno-negative and 1 for immuno-positive) on the basis of the cutoff values that were calculated from the respective ROC curves. The binary values of staining for cldn18, maspin (N), and p53 were then combined to obtain an additional score. Binary processing of multiplication scores did not impair the high sensitivity and specificity of the original scores. For the combined binary values, cutoff values of 2 or 3, denoting immune-positivity for two or three antibodies, provided the highest sensitivity and specificity (91.1 and 100 %, respectively).

In the analysis of presurgical biopsy specimens, we divided the specimens into three groups on the basis of histology (adenocarcinoma/BilIN, malignancy-undetermined atypical epithelium, and non-neoplastic epithelium), because the condition of the biopsy specimens varied as described above which made it difficult to classify them precisely into multiple stages as we did with the surgical specimens. Among the groups, the patients with a final diagnosis of “malignancy-

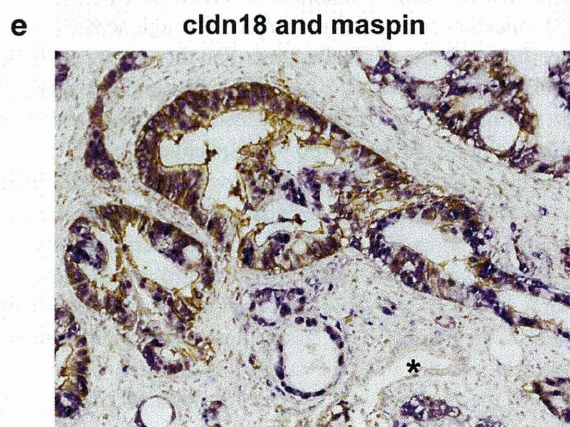


b

	adc/BillN	atypical epithelium	non-neoplasm
Score 0	0	1	15
Score 1	0	2	3
Score 2 (binary value)	7	6	1
Score 3	11	12	0
Total	18	21	19

c

adc vs non-neoplasm	Sensitivity	Specificity
Score ≥ 1	1.000	0.789
Score (binary value) ≥ 2	1.000	0.947
Score ≥ 3	0.611	1.000



d

atypical vs non-neoplasm	Sensitivity	Specificity
Score ≥ 1	0.952	0.789
Score (binary value) ≥ 2	0.857	0.947
Score ≥ 3	0.571	1.000

undetermined atypical epithelium” were not biopsy-proven but were strongly suspected of having malignant tumors based on their clinical presentation, clinical course, and imaging and examination findings. Those cases resulted in an indication for surgery and then received a definitive diagnosis of adenocarcinoma based on pathological examinations. We believe that these cases should be discussed separately from typical biopsy-proven adenocarcinoma, because a definitive diagnosis on suspected adenocarcinoma biopsy is important for treatment selection including surgical resection as early as possible. In this study, 85.7 % of malignancy-undetermined atypical epithelium (18/21) was differentiated as neoplastic.

In the present study, we included presurgical biopsy specimens with reactive/regenerative epithelium into the non-neoplastic epithelium category. We also included those with superficial spread lesions in the BilIN category as intraepithelial neoplastic lesions even though they are not usually classified this way. In presurgical endobiliary forceps biopsy specimens, a binary cutoff value of 2 was used for analysis and all 18 of the specimens with adenocarcinoma/BilIN and 1 of 19 with non-neoplastic epithelium were distinguished as neoplasms. The sensitivity and specificity were 100 and 94.7 %. These results reveal that even tiny biopsy specimens can be analyzed with high sensitivity. Of special note is the extremely high specificity, which could help to minimize indeterminate diagnoses in biopsy specimens.

In the statistical analysis of this study, there was no significant difference between BilIN-2 and BilIN-3 by using either each single marker or the panel of the three markers. To date, a number of candidate markers have been examined, some of which are expressed as early as BilIN-1, while others are only expressed from BilIN-2. For example, 25 % of BilIN-1 has been reported to carry a mutation in RAS genes [17]. BilIN-1/BilIN-2 is a neoplastic proliferation that should be followed as a precursor lesion that may progress to BilIN-3 and adenocarcinoma through multiple stages [15]. The detection of BilIN-1/BilIN-2 can be expected to identify high-risk cases, which might increase its clinical importance in the future even though further study is necessary.

We went on to perform the first reported evaluation of the efficiency of dual staining for cldn18/maspin in the diagnosis of bile duct adenocarcinoma in the surgical specimens. The difference in localization (membranous for cldn18; nuclear and cytoplasmic for maspin) and the two-colored chromogenic reaction (brown for cldn18 and blue for maspin) enables easy recognition of the markers in a single slide. The use of the cldn18/maspin double-staining strategy in our study confirmed that these markers are useful in differentiating neoplasms from non-neoplastic epithelia in surgical specimens from the biliary tract.

In conclusion, we show that immunohistochemical staining cldn18, maspin, and p53 as a panel achieves reliable distinction of biliary tract cancers and BilINs from non-neoplastic

epithelia in both surgical and biopsy specimens. This panel can serve to improve diagnostic accuracy and might aid the early diagnosis of biliary tract carcinoma and BilIN in presurgical biopsy specimens. This study is limited because it is retrospective; however, the high sensitivity and specificity observed suggests that this approach would support a diagnosis even in indeterminate cases. Further prospective and retrospective studies are needed to evaluate the practical value of this diagnostic method for clinical application.

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Conflict of interest The authors declare that they have no conflict of interest.

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Trials of vaccines for pancreatic ductal adenocarcinoma: Is there any hope of an improved prognosis?

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Abstract Pancreatic tumors are chemoresistant and malignant, and there are very few therapeutic options for pancreatic cancer, as the disease is normally diagnosed at an advanced stage. Although attempts have been made to develop vaccine therapies for pancreatic cancer for a couple of decades, none of the resultant protocols or regimens have succeeded in improving the clinical outcomes of patients. We herein review vaccines tested within the past few years, including peptide, biological and multiple vaccines, and describe the three sets of criteria used to evaluate the therapeutic activity of vaccines in solid tumors.

Keywords Pancreatic cancer · Vaccine · Immunomodulation

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States [1–3] and the fifth most common cause of such deaths in Japan [4]. Although surgical resection is considered to be the only curative therapy for pancreatic cancer, only 20 % of patients have resectable disease at the time of diagnosis [5, 6]. In addition, advanced pancreatic cancer patients exhibit a median survival time (MST) of approximately six months and a 5-year overall survival rate of less than 5 %, despite efforts to manage the tumors with chemotherapy, radiotherapy and other treatments [3, 5–8].

In 1997, Burris et al. reported that gemcitabine monotherapy is superior to fluorouracil (5-FU) monotherapy for

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Table 1 Chemotherapy for advanced pancreatic cancer

	Median survival time (months)	Overall response rate (%)	Trial name	References
Gemcitabine	5.65	5.4		J Clin Oncol 1997;15: 2403–13.
Gemcitabine + erlotinib	6.24	8.6	NCIC CTG PA.3	J Clin Oncol 2007;25: 1960–6.
FOLFIRINOX	11.1	31.6	ACCORD 11	N Engl J Med 2011;364: 1817–25.
Nab-paclitaxel + gemcitabine	8.7	29.2	MPACT trial NCT00844649	N Engl J Med 2013;369: 1691–703.
Gemcitabine +TS-1	10.1	29.3	GEST trial	J Clin Oncol 2013; 31:640–8.

treating pancreatic ductal adenocarcinoma (PDAC) [9]. Gemcitabine monotherapy has subsequently become the standard chemotherapy for PDAC, resulting in an MST of 5.65 months (Table 1). Currently, three protocols have proven to be superior to gemcitabine monotherapy. Combining gemcitabine with erlotinib improved the MST of PDAC to 6.24 months in the NCIC CTG PA3 trial [10], while combining gemcitabine with nab-paclitaxel improved the MST to 8.7 months in the MPACT trial [11]. FOLFIRINOX achieved the longest MST for PDAC (11.1 months) in the ACCORD11 trial [12], and the GEST study obtained similar clinical outcomes. S-1 is an oral fluoropyrimidine derivative that has been shown to be effective against various cancers, and a previous study found that it is at least as effective as gemcitabine against PDAC [13]. In addition, treatment with a combination of gemcitabine + S-1 has been demonstrated to result in an MST of 10.1 months [14]. Although these chemotherapies extend the survival period among PDAC patients, they also result in serious adverse events. Therefore, the optimal chemotherapy regimen for PDAC depends on the patient's performance status.

There have been numerous attempts to develop vaccine therapies for cancer over the past century [2, 3]. Although clinical trials of such vaccines have obtained promising results in specific patients, none of the tested vaccines has exhibited significant improvements in efficacy compared with established therapies. In addition, several issues must be resolved before vaccine therapies can be used in the clinical setting. Tumor-associated antigens (TAA) have been demonstrated to recognize specific human leukocyte antigens (HLA) [15]. Theoretically, the tumor lysate contains all of the antigens expressed by the tumor, and cytotoxic T lymphocytes (CTL) are capable of recognizing some of these antigens [16]. All vaccines for pancreatic cancer are based on the fact that CTL recognize TAA expressed on tumor cells and subsequently attack these cells. The question is how strongly and specifically each TAA stimulates CTL in vivo in the clinical setting. Immune tolerance can develop via various mechanisms, including the downregulation of the major histocompatibility complex (MHC) molecule expression, induction of

T cell anergy, reductions in the number of immune effectors and increases in the number of regulatory T cells [17, 18], which may explain why no cancer vaccine therapy has been established as a standard treatment for advanced PDAC. Therefore, in this study, we comprehensively reviewed the clinical outcomes of vaccine therapy against advanced PDAC.

Peptide-based vaccines developed within the past few years

MUC1

Mucin 1, cell surface associated, (MUC1) is a type I transmembrane protein containing multiple tandem repeats of a 20-amino acid sequence. Several MUC1 peptides have been tested as vaccines in the clinical setting; however, most of them have failed to activate CTL [19–21]. Ramanathan et al. [22]; Yamamoto et al. [23] injected pancreatic patients with a vaccine containing a 100-mer extracellular tandem repeat domain of MUC1 and Montanide ISA-51, and both studies obtained similar clinical responses; i.e., the authors detected cytokines (interferon (IFN)- γ or interleukin (IL)-4) and anti-MUC1 antibodies in the patients' sera but did not observe any significant clinical effects. Another recent study involving a vaccine based on a different MUC1 epitope showed similar clinical outcomes, i.e., all seven patients had progressive disease (PD), although some of the patients exhibited immunological responses, such as IFN- γ and granzyme B secretion [24].

K-RAS mutants

K-RAS mutations are frequently found in patients with PDAC. Vaccines targeting mutations in codon 12 of the K-RAS gene have been tested as treatments for advanced [25] or postoperative [26] PDAC in the clinical setting. Gjertsen et al. [21] investigated the utility of a K-RAS peptide vaccine containing granulocyte-macrophage colony-stimulating factor (GM-CSF) in 10 patients who had undergone potentially curative

resection (CTN RAS 95002) and 38 patients with advanced disease (CTN RAS 97004). In that study, one patient achieved a partial response (PR), which lasted for 28 months, and the MST of the immunological responders was 4.9 months, compared to 2.0 months for the non-responders.

Human telomerase reverse transcriptase (hTERT)

Human telomerase reverse transcriptase (hTERT) is frequently expressed in cancer cells [27]. hTERT maintains functional telomeres at the end of chromosomes, which protect against cell senescence [28]. A vaccine against pancreatic cancer containing the telomerase peptide GV1001: hTERT (611-626) and GM-CSF was examined by Bernhardt et al. [29], who found the MST of the immunological responders and non-responders to be 7.2 and 2.9 months, respectively.

Vascular endothelial growth factor receptor 2 (VEGFR2)

Vascular endothelial growth factor (VEGF) plays an important role in the progression of PDAC. The type 2 VEGF receptor (VEGFR2) is expressed in PDAC and associated with tumor neovascularization. Miyazawa et al. [30] investigated the efficacy of combined treatment consisting of PDAC with a VEGFR2-169 peptide-based vaccine and gemcitabine chemotherapy and reported that one patient achieved a PR, while the disease control rate was 67%. In addition, the MST was 7.7 months, although 15/18 patients were chemotherapy naive.

G17DT (gastrimmune)

Gastrin is expressed in PDAC and plays a role in regulating the autocrine, paracrine and endocrine systems [31]. The administration of the anti-gastrin immunogen G17DT results in increased serum antibody levels and reduced tumor growth in patients with gastrointestinal malignancies [32]. A randomized, double-blind, placebo-controlled multicenter trial of G17DT was also recently performed [33]. Although, among the intention to treat (ITT) population, no significant differences in MST were detected between the PDAC patients treated with G17DT and those given the placebo, the MST of the two groups differed significantly after excluding major protocol violators and censoring for chemotherapy.

Heat shock protein (HSP)

Heat shock protein (HSP) itself is not an immunogen; however, it acts as a chaperone or carrier of antigenic peptides and possesses a repertoire of cellular peptides for

pancreatic cancer [34]. Furthermore, HSPPC-96 (Onco-phage) has been tested as a vaccine in the adjuvant setting after complete resection of PDAC [35]. In the latter study, the MST of PDAC was reported to be 2.9 months after surgery; however, this did not result in further clinical studies because only two of 10 patients exhibited increased enzyme-linked immunospot (ELISPOT) reactivity.

Biological vaccines

Fowlpox viral vaccine

Carcinoembryonic antigen (CEA) and MUC1 are highly expressed in PDAC [36]. Viral vectors carrying CEA, MUC1 and TRICOM [a triad of costimulatory molecules: B7.1, intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 3 (LFA-3)] have been investigated as vaccines against advanced PDAC [37]. In one study, a vaccinia viral vector was used for the initial T cell priming, and a fowlpox viral vector was used for immune boosting. Although this treatment resulted in an MST of 6.3 months (1.5–21.1 months), the five patients who showed T cell responses achieved a longer survival period than the five patients who did not (15.1 and 3.9 months, respectively; $P = 0.002$) [38]. It should be noted that GM-CSF was used as a vaccine adjuvant in the latter trial (Table 2).

Live-attenuated, double-deleted (LADD) Listeria monocytogene vaccine

ANZ-100 is a live-attenuated double-deleted Listeria monocytogene strain (LADD; Lm Δ actA/ Δ inlB) found to induce a local proinflammatory response, resulting in the activation of innate and adaptive effector cells [39]. Mesothelin is expressed in PDAC and plays an important role in tumor progression [40]. CRS-207 is a LADD Lm strain that delivers mesothelin antigens into class I and II antigen-processing pathways [41]. In a study examining the utility of CRS-207 as a treatment for advanced cancer, three of the seven subjects with PDAC were long-term survivors, although the detection of a mesothelin-specific T cell response was not correlated with survival [41].

Recent vaccine therapies

WT1

Kobayashi et al. reported a retrospective analysis of 255 advanced PDAC patients who were treated with dendritic

Table 2 Peptide-based vaccines and biological vaccines for advanced pancreatic cancer

Author	Journal	Antigen peptide	Sequences	Combination	Patients	Outcome/MST
Yamamoto	Anticancer Res. 2005;25:3575–9	MUC1	10-mer extracellular tandem repeat domain: (GVTSAPDTRPAPGSTAPPAH) ₅	Montanide ISA-51	6	1/6 SD
Rong	Clin Exp Med. 2012;12:173–80	MUC1	PDTRPAPGSTAPPAHGVTS	DC cells	7	All PD
Gjertsen	Int J Cancer. 2001;92:441–50	K-ras	KLVVVGAGGVGKSALTI Asp: D Arg: R Val: V Cyc: C	GM-CSF	38	1 PR 10 SD (10.2 M; 3-23 M) 27 PD 4.9 M responders 2.0 M non-responders
Abou-Alfa	Am J Clin Oncol. 2011;34:321–5	ras12R ras12 V ras12D Wild-type ras	TEYKLVWGARGVVGKSALTIQ TEYKLVWGVGAVGKSALTIQ TEYKLVWGADGVGKSALTIQ TEYKLVWGAGGVGKSALTIQ	hGM-CSF	24	Postoperative adjuvant treatment
Bernhardt	Br J Cancer. 2006;95:1474–82	Telomerase hTERT (611–626)	GV1001; EARPALLTSRLRFIPK	GM-CSF	38	7.2 M (24 responders) 2.9 M (14 non-responders)
Miyazawa	Cancer Sci. 2010;101:433–9	VEGFR2-169	RFVPDGNRI	Gemcitabine	18	7.7 M
Gilliam	Pancreas. 2012;41:374–9	Anti-gastrin G17DT Gastrimmune	EGPWLEEEEEAYGWMDf-DT (diphtheria toxoid)	G17DT vs. placebo	152	5.0 M vs 2.8 M
Maki	Dig Dis Sci. 2007;52:1964–72	HSP HSPPC-96 (gp96, Oncophage)			10	Postoperative adjuvant treatment 2.7 Y
Kaufman	J Transl Med. 2007;5:60	MUC1 and CEA	CEA agonist peptide CAP1-6D (YLSGADLNL) MUC-1 agonist peptide P-93L (ALWGQDVTSV)	B7.1, ICAM-1, LFA-3 (TRICOM) Vaccinia virus: PANVAC-V Fowlpox virus: PANVAC-F GM- CSF	10	6.3 M
Le	Clin Cancer Res. 2012;18:858–68	Listeria vaccine ANZ- 100, CRS-207			9 vs. 17	NA

Table 3 Recently developed peptide-based vaccines and multiple vaccines for advanced pancreatic cancer

Author	Journal	Antigen peptide	Sequences	Restricted HLA	Combination	Patients	Outcome/MST
Kobayashi	Cancer Immunol Immunother. 2014;63:797–806	WT1 MUC1	CYTWNQMNL RMFPNAPYL TRPAPGSTAPPAHG- VTSAP DTRPAPGSTAP	A24:02 A02:01/02:06 Any A	DC cells OK432	255	9.9 M 10.4 M (erythema)
Nishida	J Immunother. 2014;37:105–14	WT1	CYTWNQMNL	A24:02	Weekly 1000 mg/m ² GEM	31	8.1 M 10.9 M (DTH)
Asahara	J Translation Res. 2013;11:291	KIF20A-66	KVYLRVRPLL	A2402	Montanide ISA51VG	31	4.7 M 6.1 M (reaction)
Suzuki	J Immunother. 2014;37:36–42	KIF20A-10-66	KVYLRVRPLL	A2402	Montanide ISA51VG	9	5.8 M
Geynisman	J ImmunoThera Cancer. 2013;1:8	CEA CAPI-6D	YLSGADLNL	A2	Montanide/GM-CSF	19	11.1 M
Kameshima	Cancer Sci. 2013;104:124–9	SVN2B	AYACNTSTL	A2402	Montanide/IFN-oc	6	(9.6 M)
Yutani	Oncology Reports. 2013;30:1094–100	31 vaccine peptides		A2, A24, A3, A26	Mono: 8 Chemo: 33	41	7.9 M 9.6 M (chemo)
Kimura	Pancreas. 2012;41:195–205	WT1, Her2, CEA, MUC1, CA125, autologous tumor lysate			DC cells plus LAK plus GEM and S1 OK432	49	S: 8.0 M G: 12.0 M GS + LAK: 16.9 M
Le	J Clin Oncol. 2014;32(suppl 3):Abstract 177	GVAX pancreas and CRS-207 vs. GVAX pancreas alone	Irradiated GM-CSF- secreting allogeneic pancreatic tumor vaccine (GVAX pancreas)		Cyclophosphamide	90	6.1 M vs. 3.9 M 9.7 M (3 or more rounds of vaccine therapy)

cell (DC) vaccines containing Wilms tumor 1 (WT1) and MUC1 after being recruited from seven institutions that followed a unified standard operating procedure. The MST of these patients was 9.9 months [42]. Nishida et al. also examined the utility of chemo-vaccine therapy in which a WT1-based vaccine was used in combination with the administration of 1,000 mg/m² of gemcitabine weekly. The latter regimen resulted in an MST of 8.1 months among 31 advanced PDAC patients [43]. In addition, the MST of the immunological responders in these two studies was very similar (10.4 and 10.9 months, respectively) (Table 3).

KIF20A

Kinesin family member 20A (KIF20A) plays an important role in the trafficking of molecules and organelles [44] and is one of the molecules targeted by vaccines against PDAC. A KIF20A vaccine was recently tested using different regimens, including vaccine monotherapy [45] and chemo-vaccine therapy involving gemcitabine [46], and similar MST values were reported in both studies (4.7 and 5.8 months, respectively).

Carcinoembryonic antigen (CEA)

CEA is a 180-kDa immunoglobulin-like molecule expressed on the surface of 90 % of PDAC tumor cells [47]. CAP1-6D, a modified CEA peptide, was combined with Montanide/GM-CSF to produce a vaccine against pancreatic cancer that was subsequently tested in advanced PDAC patients [48]. The MST of the 19 patients was 11.1 months, and one patient, randomized into the 0.01 mg arm, achieved a complete response (CR).

Survivin2B

Survivin is a member of the inhibitors of apoptosis (IAP) family of proteins that protect apoptotic signals by inhibiting the caspase activity [49, 50]. Hence, survivin-expressing cancer cells escape from apoptosis and do not die. Using a peptide-binding assay, we found that the survivin2B 80–88 peptide induces a strong CTL response [51]. We also examined the effects of a survivin2B 80–88 peptide-based vaccine on various cancers in the clinical setting and obtained promising outcomes. In particular, the anti-tumor effect of the survivin2B 80–88 peptide was enhanced by combining it with incomplete Freud's adjuvant and IFN- α injection. Our preliminary clinical study demonstrated a 66.6 % disease control rate in advanced PDAC patients (four of six patients) [52]. Moreover, the PDAC patients in our recent clinical phase I study exhibited an MST of 9.6 months.

Table 4 Evaluation of therapeutic activity in solid tumors

Method	WHO	RECIST	IrRC
	Sum of the products of the two longest perpendicular dimensions (bidimensional)	Sum of the longest dimensions (unidimensional)	Sum of the products of the two longest perpendicular dimensions (SPD) of all index lesions. (bidimensional)
No. of measured lesions	All lesions	Five per organ, 10 in total	Five per organ, 10 in total, and five cutaneous index lesions
CR	Disappearance of all known disease, confirmed at 4 weeks	Disappearance of all known disease, confirmed at 4 weeks	Disappearance of all known disease, confirmed at 4 weeks apart
PR	>50 % decrease in total tumor size, confirmed at 4 weeks	>30 % decrease in total tumor size, confirmed at 4 weeks	>50 % decrease in tumor burden compared with baseline in two observations at least 4 weeks apart
SD	CR, PR, and PD criteria not met	CR, PR, and PD criteria not met	CR, PR, and PD criteria not met
PD	>25 % increase in total tumor size; no CR, PR, or SD documented before increase in tumor size; new lesion (s); > 25 % increase in size of one lesion	>20 % increase in total tumor size; no CR, PR, or SD documented before increase in tumor burden; new lesion (s)	>25 % increase in tumor burden compared with nadir (at any single time point) in two consecutive observations at least 4 weeks apart

Tumor burden = SPD_{index lesions} + SPD_{new, measurable lesions}

Multiple vaccines

Personalized peptides

In a previous study, a set of 31 peptides was used to create personalized vaccines for advanced PDAC [53]. A maximum of four peptides were selected from among the 31-peptide set based on the results of HLA typing and the patients' peptide-specific IgG titers. Eight patients received vaccine monotherapy, and 31 patients received chemo-vaccine therapy. In the chemo-vaccine therapy group, gemcitabine was administered in eight patients, S-1 was administered in six patients and gemcitabine + S-1 was given in eight patients. The overall MST was 9.6 months, although that of the patients who underwent monotherapy was 7.9 months. Yanagimoto et al. reported similar clinical outcomes for chemo-vaccine therapy involving personalized vaccines and gemcitabine based on the same regimen [54]. The MST of the patients in the latter study was 9.0 months, although that of the immunological responders was 15.5 months. None of the patients in either study achieved CR (Table 3).

Autologous tumor lysate combined with lymphokine-activated killer cell therapy

Kimura et al. treated 49 PDAC patients with vaccines based on five different peptides and autologous tumor lysate, although the vaccine preparation regimens and anti-tumor therapies varied in each case [16]. Two patients achieved CR after treatment with a combination of DC cell and lymphokine-activated killer cell (LAK) therapy. The MST of the patients treated with LAK + gemcitabine and S-1 was 16.9 months, whereas that of all patients was 12.0 months. It should be noted that the survival time was calculated from the day after the first vaccination, which may have resulted in a shorter survival time (by a couple of months) than would have been obtained using the methods employed in other studies. It is very difficult to evaluate the clinical results of this study due to the effects of the different therapeutic strategies used in each case. However, the fact that multiple patients achieved CR will encourage researchers to pursue this approach further.

GVAX pancreas with CRS-207

GVAX is a series of irradiated GM-CSF-secreting allogeneic pancreatic cell lines that elicit broad antigenic responses. CRS-207 is a LADD Lm strain (Lm Δ actA/ Δ inlB) that expresses mesothelin and stimulates the innate and adaptive immune systems. A phase II randomized control trial of GVAX pancreas combined with CRS-207 versus GVAX pancreas alone was presented at the 2014

American Society of Clinical Oncology (ASCO) Gastrointestinal Cancers Symposium [55]. Interestingly, the clinical results demonstrated that both treatments had dose-dependent survival benefits. The MST of the patients who received three or more rounds of vaccine therapy was 9.7 months, and the MST of the GVAX with CRS-207 arm was longer than that of the GVAX-alone arm (6.1 vs. 3.9 months; $P = 0.01$) [56].

Evaluation of therapeutic activity in solid tumors

The response of solid tumors is evaluated using either the WHO [57] or RECIST criteria [58]. The RECIST criteria were developed because the WHO criteria are quite complex and measuring all visible lesions in two dimensions is both time consuming and subject to measuring bias [59]. However, the use of immunotherapeutic agents in cancer patients is associated with the following problems: (a) The measurable anti-tumor activity can take longer to appear during immunotherapy than during cytotoxic therapy; (b) Responses to immunotherapy can occur after the standard criteria for progressive disease (PD) have been met; (c) Discontinuing immunotherapy may not be appropriate in some cases, unless PD is confirmed; (d) Allowing for "clinically insignificant" PD (e.g., small new lesions developing in the presence of other responsive lesions) is recommended; and (e) Durable stable disease (SD) may represent the anti-tumor activity [60]. Therefore, the immune-related response criteria (irRC) were developed to evaluate the immunotherapeutic activity in solid tumors [61]. The most important aspects of the irRC criteria are that (a) new lesions are not classified as PD and (b) two consecutive observations obtained at least four weeks apart are required to diagnose PD. However, the clinical utility of the irRC remains unclear and these criteria may require further optimization [61] (Table 4).

Future research topics

Initial time point for survival assessments

The initial time point for survival assessments should be unified to allow clinical outcomes to be compared between studies. Most PDAC patients already have advanced disease at the time of diagnosis [6]. In addition, the adverse effects of chemotherapies differ markedly among the various regimens [8]. Therefore, the status of PDAC patients at the time point at which they are registered can differ both within and between clinical studies. Kobayashi et al. reported that the MST from the date of diagnosis and the MST from the first vaccination are very different (16.5 vs.

9.9 months) [42]. Therefore, MST data must be interpreted carefully.

Vaccine therapy and chemotherapy

The goal of vaccine therapy for cancer is to increase the native immunity of cancer patients. However, chemotherapy causes irreversible damage to proliferating cancer cells as well as immune cells, including T and B cells. Therefore, there is a conflict between the fundamental principles of these two treatments. Chemotherapy is currently the gold standard treatment for advanced PDAC. Although the biological mechanisms of vaccine therapy and chemotherapy conflict with each other, the anti-cancer activity of vaccine monotherapy or chemo-vaccine combination therapy should be greater than that of chemotherapy alone.

Slow clinical response to vaccine therapy

It is very hard to achieve a complete response (CR) with vaccine therapy alone. We reviewed 19 studies involving a total of 860 patients and found that CR responses were obtained in only three cases. Although none of these studies involved a large number of patients, the poor reported response rates are a concern. One of the patients who achieved a CR was administered CEA CAPI-6D + Montanide/GM-CSF therapy, while the other two were treated with WT1, Her2, CEA, MUC1, cancer antigen 125 and autologous tumor lysate vaccines combined with DC cell-based LAK therapy and chemotherapy. Immunological responses require a long time to control tumor growth and achieve remission. The primary goal of vaccine therapy is to achieve long-term SD [62]. Most previous clinical studies of PDAC involved patients with advanced disease for whom no other therapies were available. Therefore, vaccine therapy may be suitable for patients in other clinical stages or possibly a useful postoperative adjuvant therapy. The main advantage of vaccine therapy is that it has few adverse effects, although it has also demonstrated minimal clinical effects in previous trials. We are currently conducting a phase II study of the survivin2B 80–88 peptide + Montanide + IFN- β as a treatment for PDAC (SUCCESS, Study of Unresectable CanCER with Survivin-2B peptide vaccine in Sapporo: UMIN000012146), in which half of the required patients have been recruited. The clinical results of the SUCCESS phase II study will be reported by the end of next year.

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Heat shock protein 90 targets a chaperoned peptide to the static early endosome for efficient cross-presentation by human dendritic cells

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The presentation of an exogenous antigen in a major histocompatibility complex class-I restricted fashion to CD8⁺ T cells is called cross-presentation. Heat shock proteins (HSPs) such as Hsp70, gp96, and Hsp90 have been shown to elicit efficient CTL responses by cross-presentation through an as-yet entirely unknown mechanism. Hsp90 is the most abundant cytosolic HSP and is known to act as a molecular chaperone. We have shown that a tumor antigen peptide complexed with Hsp90 could be cross-presented by dendritic cells (DCs) through an endosomal pathway in a murine system. However, it has not been determined whether human DCs also cross-present an Hsp90–peptide complex and induce peptide-specific CTLs. In this study, we found that an Hsp90–cancer antigen peptide complex was efficiently cross-presented by human monocyte-derived DCs and induced peptide-specific CTLs. Furthermore, we observed that the internalized Hsp90–peptide complex was strictly sorted to the Rab5⁺, EEA1⁺ static early endosome and the Hsp90–chaperoned peptide was processed and bound to MHC class I molecules through an endosome-recycling pathway. Our data indicate that targeting of the antigen to a “static” early endosome by Hsp90 is essential for efficient cross-presentation.

The generation of specific CD8⁺ CTLs is thought to play a key role in the control of virus-infected cells and tumors. However, immunization with peptides or recombinant proteins generally fails to elicit CTLs because an immunized antigen (Ag) acts as an exogenous Ag. Generally, an exogenous Ag enters the MHC class II pathway and is presented to CD4⁺ T cells in the context of MHC class II molecules. However, professional Ag-presenting cells, particularly DCs, can take up exogenous Ag and present them on their MHC class I molecules. This process is called cross-presentation and plays an important role in the control of virus-infected cells and tumor growth.⁽¹⁾ There are two pathways of cross-presentation: cytosolic (endoplasmic reticulum–Golgi-dependent) and vacuolar (endosomal) pathways.^(2,3) One of the reasons for inefficiency of a vaccine strategy is that the vaccine Ag is usually administered as an exogenous Ag, and it is therefore difficult to introduce the vaccine Ag into the cross-presentation pathway. To overcome this problem, various methods have been developed to target an exogenous Ag into the endogenous MHC class I-restricted pathway. In our previous studies, we showed that extracellular Hsp90–peptide complexes are efficiently cross-presented through the endosome-recycling pathway.⁽⁴⁾ In this Hsp90-mediated cross-presentation, the receptor-dependent en-

docytosed 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 resulting in the expression of 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*. However, how the Hsp90–peptide complex is sorted out after receptor-dependent endocytosis remains unclear. In the present work, we found that Hsp90 complexed with a human tumor Ag peptide derived from survivin-2B^(5,6) is cross-presented by human Mo-DCs resulting in the stimulation of peptide-specific CTLs. In addition, we found that Hsp90 targets a chaperoned Ag peptide into the “static” early endosome within Mo-DCs, resulting in cross-presentation of the antigenic peptide through the recycling pathway.

Materials and Methods

The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation,

Sapporo Medical University (Sapporo, Japan). The patients and their families as well as healthy donors gave informed consent for the use of blood samples in our research.

Patient treatment. The patients were vaccinated with survivin-2B₈₀₋₈₈ (1 mg) plus Montanide ISA 51 (1 mL; Seppic, Paris, France) s.c. four times at 14-day intervals. In addition, IFN- α (3 000 000 IU; Dainippon-Sumitomo Pharmaceutical Co., Osaka, Japan) was given s.c. twice a week close to the site of vaccination. Hematological examinations were carried out before and after each vaccination.

Induction of human monocyte-derived immature dendritic cells. Autologous monocytes were purified from PBMCs from each patient that were isolated using Lymphoprep (Nycomed, Oslo, Norway). Monocytes (1×10^4 /well) in a 24-well plate were cultured in complete RPMI-1640 with 10% FCS and GM-CSF (1000 U/mL) and IL-4 (1000 U/mL) for 7 days. The medium with GM-CSF and IL-4 was gently replaced on day 2 and 4. Human recombinant GM-CSF was a kind gift from Kirin (Tokyo, Japan). Human recombinant IL-4 was purchased from Invitrogen (Carlsbad, CA, USA).

Peptides and proteins. The following peptides were used (underlined sequences representing the precise MHC class I-binding epitope): survivin-2B₈₀₋₈₈ (AYACNTSTL); and survivin-2B₇₅₋₉₃ (GPGTVAYACNTSTLGGRGG). All peptides were purchased from Sigma-Genosys (Ishikari, Japan). Human Hsp90 was purchased from StressGen (Ann Arbor, MI, USA). Human LDL was purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored at 20 mg/mL in PBS at -80°C .

Antibodies. Confocal laser microscopy was used to detect organelles with specific antibodies: an anti-Rab5 pAb (MBL, Nagoya, Japan) and EEA1 (Abcam, Cambridge, MA, USA) for early endosomes, and anti-LAMP-1 pAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for late endosomes/lysosomes. Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA) was used for labeling Hsp90 and LDL.

Generation of Hsp90-peptide complex *in vitro*. As previously described,⁽⁴⁾ Hsp90 was mixed with survivin-2B₇₅₋₉₃ (GPGTVAYACNTSTLGGRGG) in a 50:1 peptide: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.

Establishment of survivin-2B₈₀₋₈₈-specific CTL clone. We generated survivin-2B₈₀₋₈₈-specific CTL clones from a patient with colon cancer (patient 1 in Table 1). After the fourth vaccination, PBMCs were isolated from blood samples by Ficoll-Conray density gradient centrifugation. Phytohemagglutinin blasts were derived from PBMCs by culturing in AIM V medium (Invitrogen) containing 10% human serum, IL-2 (100 U/mL; Takeda Pharmaceutical Co., Osaka, Japan), and PHA (1 mg/mL; Wako Chemicals, Osaka, Japan) for 3 days, followed by washing and cultivation in the presence of IL-2 (100 U/mL) for 4 days. HLA-A*2402-survivin-2B₈₀₋₈₈ peptide tetramer-positive (MBL) CTLs were sorted and subsequently cloned to single cells using FACSaria (Becton Dickinson, San Jose, CA, USA). Survivin-2B₈₀₋₈₈-specific CTL clones were restimulated with survivin-2B₈₀₋₈₈ peptide-pulsed PHA blasts every 7 days in AIM V medium supplemented with 50 U/mL IL-2.

***In vitro* cross-presentation assay.** Human Mo-DCs (1×10^5) were pulsed with Hsp90 (400 $\mu\text{g}/\text{mL}$), survivin-2B₇₅₋₉₃ (400 $\mu\text{g}/\text{mL}$) alone, a complex of Hsp90 (100 or 400 $\mu\text{g}/\text{mL}$) and survivin-2B₇₅₋₉₃ (100 or 400 $\mu\text{g}/\text{mL}$), a simple mixture of both or survivin-2B₈₀₋₈₈ (400 $\mu\text{g}/\text{mL}$) for 2 h at 37°C in 100 μL Opti-MEM and then 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 survivin-2B peptide-specific CTL clone. Activation of CTLs was measured as IFN- γ production using ELISA. In a dose titration assay, Mo-DCs (1×10^5) were loaded with various doses of survivin-2B₈₀₋₈₈ peptide or Hsp90-precursor peptide (survivin-2B₇₅₋₉₃) complex for 2 h in 100 μL Opti-MEM and fixed with 0.01% glutaraldehyde. The cells were washed and cultured overnight with 1×10^5 survivin-2B₈₀₋₈₈-peptide-specific CTL clone. Interferon- γ in the culture supernatant was measured using ELISA.

***In vitro* stimulation of PBMCs with Mo-DC loaded with Hsp90-precursor peptide complex.** Peripheral blood mononuclear cells were isolated from eight patients suffering from various types of cancer who had been vaccinated with survivin-2B peptide in our clinical study.^(7,8) These patients' PBMCs were shown to contain survivin-2B-specific CD8⁺ T cells. The PBMCs were stimulated with Mo-DCs loaded with survivin-2B₈₀₋₈₈ (400 $\mu\text{g}/\text{mL}$), Hsp90 (400 $\mu\text{g}/\text{mL}$), survivin-2B₇₅₋₉₃ (400 $\mu\text{g}/\text{mL}$), and Hsp90 (100 or 400 $\mu\text{g}/\text{mL}$)-survivin-2B₇₅₋₉₃ (100 or 400 $\mu\text{g}/\text{mL}$) complex in AIM V medium (Life Technologies, Grand Island, NY, USA) containing 10% human serum. Interleukin-2 was added at a final concentration of 50 U/mL on days 2, 4, and 6. On day 7 of culture, the PBMCs were analyzed by tetramer staining and ELISPOT assay.

Assessment of stimulation of Ag-specific CTLs using tetramer assay. The FITC-labeled HLA-A*2402-HIV peptide (RYL-RDQQL) and PE-labeled HLA-A*2402-survivin-2B₈₀₋₈₈ peptide tetramers were purchased from MBL. For flow cytometric analysis, PBMCs, which were stimulated *in vitro* as described above, were stained with HIV tetramer or survivin-2B tetramer at 37°C for 20 min. Then a PE-Cy5-conjugated anti-CD8 antibody (eBioscience, San Diego, CA, USA) was added at 4°C for 30 min. Cells were washed twice with PBS. After washing, cells were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry using FACSCalibur and CellQuest software (Becton Dickinson). CD8⁺ living cells were gated and cells labeled with survivin-2B tetramer were referred to as tetramer-positive cells. The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8⁺ T cells.

Assessment of stimulation of Ag-specific CTLs using ELISPOT assay. The ELISPOT plates were coated sterilely overnight with anti-IFN- γ capture antibody (BD Biosciences, San Jose, CA, USA) at 4°C . The plates were then washed once and blocked with AIM V medium containing 10% human serum for 2 h at room temperature. CD8-positive T cells separated from patients' PBMCs (5×10^3 cells/well), which were stimulated *in vitro* as described above, were then added to each well along with HLA-A24-transfected T2 (T2-A24) cells (5×10^4 cells/well) that had been preincubated with survivin-2B₈₀₋₈₈ (10 $\mu\text{g}/\text{mL}$) or HIV with an HIV peptide as a negative control. After incubation in a 5% CO₂ humidified chamber at 37°C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN- γ antibody (R&D Systems, Minneapolis, MN, USA) and HRP-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Jena, Germany).

Immunocytological localization of Hsp90-survivin-2B₇₅₋₉₃ peptide complex. Heat shock protein 90 and LDL were conjugated with Alexa Fluor 594 (Molecular Probes) according to the manufacturer's instructions. Monocyte-derived DCs were incubated at 37°C with Alexa Fluor 594-labeled Hsp90 (20 μg) complexed with survivin-2B₇₅₋₉₃ peptide (20 μg) for 1 h.

Table 1. Quantitation of survivin-2B-specific CD8⁺ T cells by tetramer assay

Patient no.	Tumor	Survivin-2B ₈₀₋₈₈ -specific CD8 ⁺ T cell frequency (tetramer staining)						Effect
		<i>In vitro</i> stimulation	(-)	Survivin-2B ₈₀₋₈₈	Hsp90	Survivin-2B ₇₅₋₉₃	Hsp90-survivin-2B ₇₅₋₉₃	
1	Colon		0.06	4.87	4.47	3.24	8.47	††
2	Colon		0.32	2.87	0.77	4.01	1.30	No
3	Pancreas		0.70	6.27	1.70	1.88	6.64	††
4	Pancreas		0.48	4.56	0.84	1.28	3.02	†
5	Ampulla of Vater		1.27	4.60	0.97	2.24	6.50	††
6	Breast		3.59	3.78	3.00	3.06	3.82	††
7	Breast		3.98	3.91	1.94	2.28	6.19	††
8	Breast		2.76	3.92	2.91	2.08	6.07	††

†Frequency of survivin-2B-specific CD8⁺ T cells stimulated with heat shock protein 90 (Hsp90)-survivin-2B₇₅₋₉₃ peptide complex was increased compared with stimulation with survivin-2B₇₅₋₉₃ precursor peptide. ††Frequency of survivin-2B-specific CD8⁺ T cells stimulated with Hsp90-survivin-2B₇₅₋₉₃ peptide was increased compared with stimulation with both survivin-2B₈₀₋₈₈ peptide and survivin-2B₇₅₋₉₃ peptide. (-), negative control; No, no effect.

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 and anti-LAMP-1 pAb for late endosomes followed by Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes) or anti-mouse IgG (Molecular Probes) and then visualized with a Bio-Rad MRC1024ES confocal scanning laser microscope system (Bio-Rad, Richmond, CA, USA). For evaluation of colocalization, a single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 150 cells (50 cells from three independent experiments) were analyzed.

Inhibition studies. Monocyte-derived DCs were pre-incubated with chloroquine (Sigma-Aldrich) or primaquine (ICN Bio-medicals, Irvine, CA, USA) at 37°C for 2 h, and then loaded with survivin-2B₈₀₋₈₈ peptide alone or Hsp90-precursor peptide (survivin-2B₇₅₋₉₃) complex for 2 h. The Mo-DCs were then fixed, washed, and cultured overnight with survivin-2B₈₀₋₈₈-specific CTL clone. Activation of CTLs was measured as IFN- γ production using ELISA.

Statistical analysis. All experiments were independently carried out three times in triplicate. Results are shown as means + SEM. Comparisons between two groups were performed using Student's *t*-test, with a *P*-value < 0.05 considered to be statistically significant.

Results

Heat shock protein 90-survivin-2B₇₅₋₉₃ peptide complex is cross-presented by Mo-DCs *in vitro*. We first examined whether human Hsp90 facilitated cross-presentation of the chaperoned precursor peptide by human Mo-DCs. The Mo-DCs were pulsed with Hsp90 alone, the survivin-2B₇₅₋₉₃ precursor peptide alone, a simple mixture of both, a complex of them generated *in vitro* at double concentration, or survivin-2B₈₀₋₈₈ peptide (for positive control) for 2 h at 37°C and then fixed, washed, and cultured with survivin-2B₈₀₋₈₈-specific CTL clone. The Hsp90-survivin-2B₇₅₋₉₃ precursor peptide complex elicited a significant amount of IFN- γ production both at 100 and 100 μ g/mL, whereas Hsp90 alone, survivin-2B₇₅₋₉₃ precursor peptide alone, or a simple mixture of both did not induce IFN- γ production by CTLs (Fig. 1a). Strikingly, IFN- γ production induced by Hsp90-survivin-2B precursor peptide complex was much greater than that induced by survivin-2B peptide. These results indicated that cross-presentation of survivin-2B-derived

peptide was enhanced when an exogenous precursor peptide was complexed to Hsp90. To confirm these observations, we compared the efficacy of CTL activation between survivin-

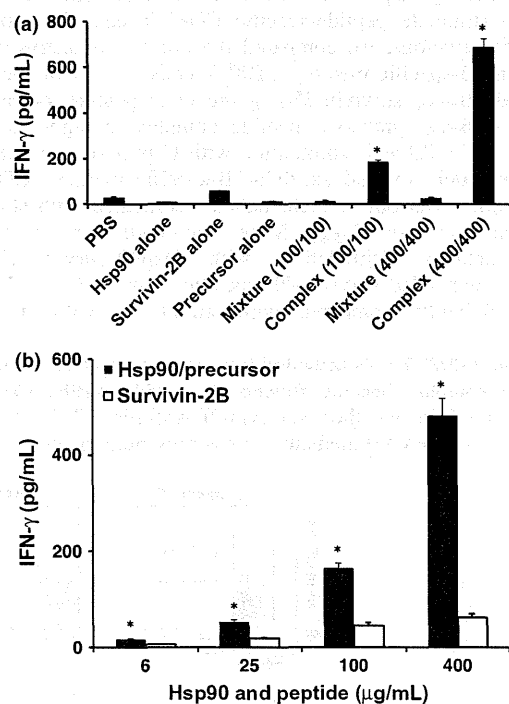


Fig. 1. Cross-presentation of heat shock protein 90 (Hsp90)-chaperoned peptides by human monocyte-derived dendritic cells (Mo-DCs). (a) Human Mo-DCs (1×10^5) were pulsed with Hsp90 (400 μ g/mL), precursor peptide survivin-2B₇₅₋₉₃ (400 μ g/mL) alone, a complex of Hsp90 (100 or 400 μ g/mL) and survivin-2B₇₅₋₉₃ (100 or 400 μ g/mL), a simple mixture of both, or survivin-2B₈₀₋₈₈ peptide (for positive control) for 2 h at 37°C and then fixed with 0.01% glutaraldehyde, washed, and cultured with survivin-2B₈₀₋₈₈-specific CTL clone (1×10^5 /well). Activation of CTLs was measured as γ -interferon (IFN- γ) production using ELISA. (b) Mo-DCs (1×10^5) were loaded with various doses of survivin-2B₈₀₋₈₈ peptide (6, 25, 100, and 400 μ g/mL) or Hsp90-survivin-2B₇₅₋₉₃ precursor peptide complex (6/6, 25/25, 100/100, and 400/400 μ g/mL) for 2 h in 100 μ L Opti-MEM and fixed with 0.01% glutaraldehyde. The cells were washed and cultured overnight with 1×10^5 survivin-2B₈₀₋₈₈-specific CTL clone. Activation of CTLs was measured as IFN- γ production using ELISA. Data are shown as means + SEM of three independent experiments. **P* < 0.01.

2B₈₀₋₈₈ peptide and Hsp90–survivin-2B₇₅₋₉₃ precursor peptide complex in a dose titration assay (Fig. 1b). We observed that stimulation of the survivin-2B₈₀₋₈₈-specific CTL clone with Hsp90–survivin-2B₇₅₋₉₃ precursor peptide complex was more effective than stimulation with survivin-2B₈₀₋₈₈ peptide at any dose.

Peptide-specific precursor CTLs are activated by cross-presentation of Hsp90–peptide complex. As we had shown that the Hsp90–survivin-2B₇₅₋₉₃ precursor peptide complex was efficiently cross-presented, we next examined whether cross-presentation of Hsp90–peptide complex could activate and expand peptide-specific memory CD8⁺ T cells from patients who had been vaccinated with survivin-2B peptide with incomplete Freund's adjuvant. Activated and expanded survivin-2B-specific CD8⁺ T cells were detected by tetramer staining. As shown in Figure 2, the survivin-2B₇₅₋₉₃ precursor peptide chaperoned by Hsp90 was able to activate and expand survivin-2B-specific memory CD8⁺ T cells more vigorously than was the precursor peptide alone. Interestingly, peptide-specific T-cell frequency was higher when stimulated with Hsp90–survivin-2B₇₅₋₉₃ precursor peptide complex than that with survivin-2B₈₀₋₈₈ peptide, indicating that a long peptide chaperoned by Hsp90 was efficiently cross-presented and was able to stimulate peptide-specific CD8⁺ T cells. To confirm these observations, we compared the efficacy of activation of survivin-2B-specific memory CD8⁺ T cells by stimulation with survivin-2B₈₀₋₈₈, survivin-2B₇₅₋₉₃ precursor peptide, or Hsp90–survivin-2B₇₅₋₉₃ precursor peptide complex in eight patients. As shown in Table 1, stimulation with Hsp90–survivin-2B₇₅₋₉₃ complex could expand survivin-2B-specific memory CD8⁺ T cells from seven out of eight patients compared with stimulation with survivin-2B₇₅₋₉₃. More importantly, in six out of eight patients, stimulation with Hsp90–survivin-2B₇₅₋₉₃ complex expanded survivin-2B-specific memory CD8⁺ T cells more efficiently compared with stimulation with survivin-2B₈₀₋₈₈.

Memory CD8⁺ T cells activated by cross-presentation of Hsp90–peptide complex become functional peptide-specific CTLs. To further confirm whether survivin-2B-specific CD8⁺ T cells activated by Hsp90-mediated cross-presentation were func-

tional or not, we carried out an ELISPOT assay using CD8⁺ T cells from a patient who had been vaccinated with survivin-2B peptide with incomplete Freund's adjuvant. Figure 3 shows that stimulation of CD8⁺ T cells from the patient with Hsp90–survivin-2B₇₅₋₉₃ precursor peptide complex clearly increased functionally positive survivin-2B-specific CD8⁺ T cells compared with stimulation with survivin-2B₇₅₋₉₃ precursor peptide or survivin-2B₈₀₋₈₈ peptide. When CD8⁺ T cells from the patient were stimulated with Hsp90 (400 µg/mL)–precursor peptide (400 µg/mL) complex, the number of IFN-γ-positive spots was less than that of CD8⁺ T cells stimulated with Hsp90 (100 µg/mL)–precursor peptide (100 µg/mL) complex. These results were due to the formation of fused large spots that were observed when stimulated with Hsp90 (400 µg/mL)–precursor peptide (400 µg/mL) complex and therefore the number of ELISPOT counted became smaller than that of Hsp90 (100 µg/mL)–precursor peptide (100 µg/mL) complex. These findings indicated that Hsp90–peptide complex is efficiently cross-presented by human Mo-DCs and is capable of stimulating peptide-specific CTLs.

Immunocytological localization of Hsp90–survivin2B₇₅₋₉₃ peptide complex. For further support of the above-described results, we investigated the intracellular routing of Hsp90 after uptake of it in DCs, using confocal laser microscopy. The Mo-DCs were incubated with Alexa 594-labeled Hsp90–survivin2B₇₅₋₉₃ peptide complex for 1 h. Following incubation, the cells were fixed and stained with antibodies against markers for organelle structures including EEA1, Rab5, and LAMP-1. Alexa 594-labeled Hsp90–peptide complex was detected in EEA1⁺ and Rab5⁺-early endosomes but not in lysosomes (Fig. 4a). Quantitative analysis of the colocalization between the exogenous Hsp90–peptide complex and Rab5, EEA1, and LAMP1 revealed average colocalization incidences of 78.0%, 88.7%, and 7.3%, respectively, providing further evidence that the exogenous Hsp90–peptide complex was delivered to the endosome-recycling pathway (Fig. 4b). We also examined the dynamics of Alexa 594-labeled LDL as a positive control protein for the dynamic early endosomal pathway (Fig. 5). Alexa594-labeled soluble LDL localized to the Rab5⁺-early endosome as well as the LAMP-1⁺-late endosome/lysosome,

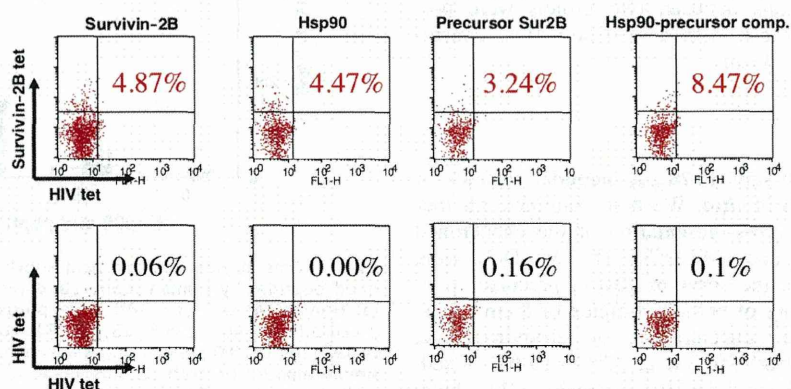


Fig. 2. Peptide-specific precursor CTLs were activated by cross-presentation of heat shock protein 90 (Hsp90)–peptide complex. PBMCs were isolated from patient 1 suffering from colon cancer (Table 1) who had been vaccinated with survivin-2B₈₀₋₈₈ peptide in our clinical study. The patient's PBMCs were shown to contain the survivin-2B-specific CD8⁺ T cells. PBMCs were stimulated with human monocyte-derived dendritic cells loaded with survivin-2B₈₀₋₈₈ (400 µg/mL), Hsp90 (400 µg/mL), survivin-2B₇₅₋₉₃ precursor peptide (400 µg/mL), and Hsp90 (400 µg/mL)–survivin-2B₇₅₋₉₃ precursor peptide (400 µg/mL) complex in AIM V medium containing 10% human serum and interleukin-2 (50 U/mL) for 7 days. The stimulated PBMCs were stained with HIV tetramer (tet) or survivin-2B tetramer at 37°C for 20 min. Then a phycoerythrin-Cy5-conjugated anti-CD8 antibody was added at 4°C for 30 min. Cells were washed twice with PBS. After washing, cells were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry using FACSCalibur and CellQuest software. CD8⁺ living cells were gated, and cells labeled with survivin-2B tetramer were referred to as tetramer-positive cells. The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8⁺ cells. Data are shown as means + SEM of three independent experiments. **P* < 0.01.

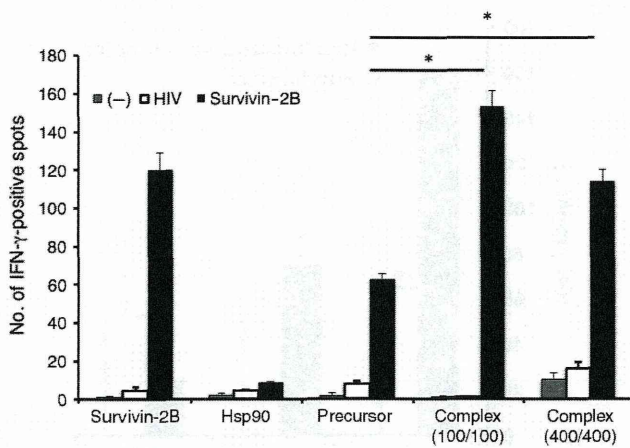


Fig. 3. Memory CD8⁺ T cells activated by cross-presentation of heat shock protein 90 (Hsp90)–peptide complex became functional peptide-specific CTLs. CD8⁺ T cells separated from PBMCs (5×10^3 cells/well) from patient 1 (Table 1) were stimulated with human monocyte-derived dendritic cells loaded with survivin-2B₈₀₋₈₈ (400 μ g/mL), Hsp90 (400 μ g/mL), precursor peptide survivin-2B₇₅₋₉₃ (400 μ g/mL), and Hsp90 (100 or 400 μ g/mL)–survivin-2B₇₅₋₉₃ (100 or 400 μ g/mL) complex, were added to each well along with HLA-A24-transfected T2 (T2-A24) cells (5×10^4 cells/well) that had been preincubated with survivin-2B₈₀₋₈₈ (10 μ g/mL) or HIV in the HIV peptide as a negative control (-). After incubation in a 5% CO₂ humidified chamber at 37°C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human γ -interferon (IFN- γ) antibody and HRP-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT. Data are shown as means + SEM of three independent experiments. * $P < 0.01$.

but not to the EEA1⁺-compartment, thus indicating the dynamic endosomal pathway. These results indicated that the Hsp90–peptide complex was sorted into the static endosomal pathway, not the dynamic endosomal pathway, within human Mo-DCs. In contrast, the soluble LDL protein, which underwent degradation, was translocated to the dynamic endosomal pathway. These results suggested that targeting to the “static” early endosome was required for efficient cross-presentation by Mo-DCs.

Heat shock protein 90–peptide complex is cross-presented by human DCs through an endosome-recycling pathway. We then examined whether Hsp90–precursor peptide complex was cross-presented by human Mo-DCs through an endosomal pathway after targeting to the static early endosome. We used chloroquine for inhibition of endosomal acidification and primaquine for inhibition of the membrane recycling pathway. As shown in Figure 6(a), Mo-DCs that were pre-incubated with increasing concentrations of chloroquine completely blocked cross-presentation of Hsp90–survivin-2B₇₅₋₉₃ precursor peptide complex but had no substantial effect on survivin-2B₈₀₋₈₈ peptide presentation. These results indicated that cross-presentation of Hsp90–precursor peptide complex depended on endosomal acidification, possibly including proteolysis by endosomal proteases. Moreover, Mo-DC incubated with primaquine could not present the Hsp90-chaperoned precursor peptide-derived survivin-2B₈₀₋₈₈ peptide to CTL (Fig. 6b). These results indicated that the Hsp90-chaperoned precursor peptide or processed peptide entered recycling endosomes and were transferred onto recycling MHC class I molecules.

Discussion

It has been shown that immunization with tumor-derived HSPs or HSPs complexed with an Ag peptide/protein elicits tumor-

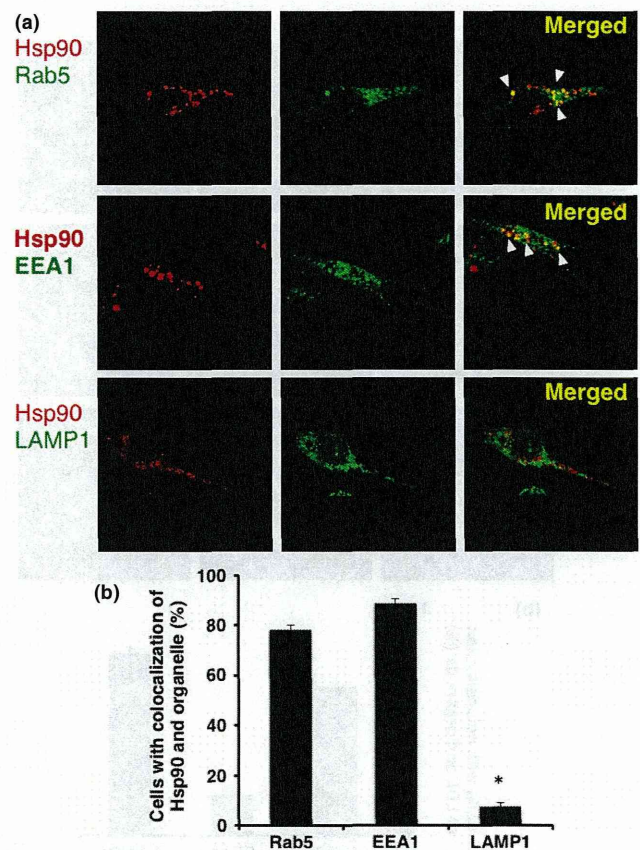


Fig. 4. Heat shock protein 90 (Hsp90)–survivin-2B₇₅₋₉₃ precursor peptide complex localized to static early endosomes within human monocyte-derived dendritic cells (Mo-DCs). (a) Human Mo-DCs were incubated at 37°C with Alexa 594-labeled Hsp90–survivin-2B₇₅₋₉₃ peptide complex for 1 h and then washed and fixed. Organelles were stained with an anti-EEA1 mAb for early endosomes, anti-Rab5 polyclonal antibody for early endosomes, and anti-LAMP-1 polyclonal antibody for late endosomes/lysosomes followed by Alexa 488-conjugated goat anti-rabbit IgG or anti-mouse IgG and were visualized with confocal laser microscopy. Arrowheads indicate colocalization of the internalized Hsp90–survivin-2B₇₅₋₉₃ peptide complex and each organelle. (b) To quantify the percentage of colocalization, a single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 150 cells (50 cells from three independent experiments) were analyzed. Data are shown as means + SEM of three independent experiments. * $P < 0.01$.

or Ag-specific CD8⁺ T cell responses.^(1,9) Importantly, it has been shown that Hsp70–Ag and gp96–Ag complexes facilitate Ag presentation in association with MHC class I molecules.^(10–13) Recently, we^(3,4) and Calderwood’s group⁽¹⁴⁾ have shown that Hsp90 also acted as an excellent navigator for associated antigens to enter the cross-presentation pathway in the murine system. We here showed that human Hsp90–cancer Ag peptide complex was efficiently cross-presented by human Mo-DCs. These results hold promise for the development of a safe and efficient immunomodulator for cancer immunotherapy. More importantly, we showed that translocation of the Hsp90–Ag complex into the static early endosome after endocytosis was crucial for efficient cross-presentation. It has been shown that the pathway for cross-presentation is comprised of two distinct intracellular routes, a proteasome–TAP-dependent pathway and an endosome-recycling pathway.^(2,3) Recent studies have revealed the pathway in which peptide exchange onto recy-