

They were then frozen and stored at  $-80^{\circ}\text{C}$ . As needed, frozen PBMC were thawed and incubated in the presence of  $30\ \mu\text{g}/\text{mL}$  survivin-2B80-88 in AIM-V medium containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of  $50\ \text{U}/\text{mL}$  1 h, 2 days, 4 days and 6 days after the addition of the peptide. On day 7 of culture, the PBMC were analyzed by tetramer staining and ELISPOT assay.

**Tetramer staining.** FITC-labeled HLA-A\*2402-human immunodeficiency virus (HIV) peptide (RYLRDQQLL) and PE-labeled HLA-A\*2402-survivin-2B8-88 peptide tetramers were purchased from MBL, Inc. (Nagoya, Japan). For flow cytometric analysis, PBMC, which were stimulated *in vitro* as above, were stained with the PE-labeled tetramer at  $37^{\circ}\text{C}$  for 20 min, followed by staining with a FITC-conjugated anti-CD8 mAb (Beckton Dickinson Biosciences, San Jose, CA, USA) at  $4^{\circ}\text{C}$  for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was performed using FACSCalibur and CellQuest software (Beckton Dickinson Biosciences, San Jose, CA, USA). The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells.<sup>(8,9,11)</sup>

**ELISPOT assay.** ELISPOT plates were coated sterilely overnight with an IFN- $\gamma$  capture antibody (Beckton Dickinson Biosciences) at  $4^{\circ}\text{C}$ . 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' PBMC ( $5 \times 10^3$  cells/well), which were stimulated *in vitro* as above, were then added to each well along with HLA-A24-transfected CIR cells (CIR-A24) ( $5 \times 10^4$  cells/well), which had been preincubated with or without survivin-2B80-88 (10 mg/mL) or with a HIV peptide as a negative control. After incubation in a 5%  $\text{CO}_2$  humidified chamber at  $37^{\circ}\text{C}$  for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN- $\gamma$  antibody and horseradish peroxidase-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Jena, Germany). In the present study, positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFN- $\gamma$ -positive spots compared with HIV peptide-specific CD8 T cell spots, whereas negative (-) represents a less than twofold increase.

**Single-cell cloning and functional assessment of tetramer-positive CTL.** Survivin-2B80-88 peptide tetramer-positive CTL were sorted and subsequently cloned to single cells using FACS (Aria II Special Order, BD, Houston, TX, USA). The peptide-specific cytotoxicity of each of these CTL was determined by pulsing T2A24 cells<sup>(8,9,11,20)</sup> with survivin-2B80-88 or HLA-A\*2402 HIV (RYLRDQQLL) peptides. These CTL were also

assessed for live tumor cell cytotoxicity against LK79 (survivin-2B positive and HLA-A24 positive), A549 (survivin-2B positive, HLA-A24 negative) and K562 (survivin-2B negative and HLA-A24 negative) target cells.

## Results

**Patient profiles and safety.** In the first protocol with the survivin-2B80-88 peptide plus IFA, five patients were enrolled in the study (Table 1). None of the treatment interruptions was due to adverse effects of the vaccination. These five patients received the complete regimen including four vaccinations and were evaluated (Fig. 1). They consisted of three men and two women, whose age range was 50–76 years.

In the second protocol with the survivin-2B80-88 peptide plus IFA and IFN $\alpha$ , eight patients were enrolled in the study (Table 2). In this protocol, there were no patients who dropped out because of adverse events due to the vaccination. They consisted of four men and four women, whose age range was 33–76 years.

With respect to safety, the vaccination was well tolerated in all patients in both vaccination protocols. In patients vaccinated with the survivin-2B80-88 peptide plus IFA, no adverse events were observed during or after vaccination except for induration at the injection site. In the second protocol, the survivin-2B80-88 peptide plus IFA and IFN $\alpha$ , approximately half of the patients had a fever reaching almost  $39^{\circ}\text{C}$  after the vaccination, possibly due to the action of IFN $\alpha$ . No other severe adverse events were observed during or after vaccination.

**Clinical responses.** As shown in Table 1, vaccination with survivin-2B80-88 plus IFA was given to five colorectal cancer patients. The post-vaccination CEA values of four patients were increased compared with the pre-vaccination values. In the other patient (No. 5), the CEA value remained almost the same, although it was beyond the upper limit during the vaccination. As for tumor size, only one patient was considered to have SD, whereas the other four patients were considered to have PD. These outcomes suggested that vaccination with this first protocol was ineffective for clinical responses.

Table 2 summarizes the clinical outcomes for the eight patients in the second protocol with survivin-2B80-88 plus IFA and IFN $\alpha$ . In some patients, particularly No. 6, the post-vaccination CEA value was clearly decreased compared with the pre-vaccination value, and was within the normal limit. Other patients such as Nos 2 and 3 also had decreased post-vaccination levels of CEA, although not so large. As for tumor size evaluated by CT, four patients (Nos 1, 2, 3 and 6) were considered to have SD, but the other four patients (Nos 4, 5, 7 and 8) had PD.

**Table 1. Summary of profiles of advanced colorectal cancer patients enrolled in the present study and clinical and immunological responses to vaccination with survivin-2B80-88 peptide and IFA**

Patient no.	Age/sex	Adverse effects	Tumor markers pre-/post- (CEA ng/mL)	CT evaluation†	Survivin-2B80-88 peptide		
					Tetramer staining‡		ELISPOTS
					Pre-/post-	% increase	
1	76/M	No	13/20	PD	192/103	53.6	-
2	59/M	No	369/463	SD	13/16	123.1	-
3	60/F	No	685/1010	PD	60/80	133.3	-
4	72/M	No	55/64	PD	11/4	36.4	-
5	50/F	No	8/7	PD	127/97	76.4	+

†Evaluation of CT images was done by the following: PD, progressive disease; SD, stable disease. ‡CTL frequencies of pre- and post-vaccinated patients were assessed with a HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with a HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer used as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTL among  $10^4$  CD8 T cells is shown. §Positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFN $\gamma$ -positive spots compared with HIV peptide-specific CD8 T cell spots, whereas negative (-) means a less than twofold increase. CEA, carcinoembryonic antigen; CT, computed tomography; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunosorbent spot; HIV, human immunodeficiency virus; IFA, incomplete Freund's adjuvant; IFN, interferon.

**Table 2. Summary of profiles of advanced colorectal cancer patients enrolled in the present study and clinical and immunological responses to vaccination with survivin-2B80-88 peptide, IFA and IFN $\alpha$**

Patient no.	Age/sex	Adverse effects	Tumor markers pre-/post- (CEA ng/mL)	CT evaluation†	Survivin-2B80-88 peptide		
					Tetramer staining‡		ELISPOT§
					Pre-/post-	% increase	
1	5/M	Leukopenia	Not determined	SD	53/98	184.9	–
2	62/M	No	5/3	SD	17/33	194.1	–
3	65/F	Fever	561/441	SD	11/98	890.9	+
4	72/M	No	6/8	PD	69/81	117.4	+
5	33/F	Fever	4655/8651	PD	8/24	300.0	+
6	44/F	Edema, Fever	12/2	SD	5/76	1520.0	+
7	53/F	Fever	82/117	PD	4/555	13 875.0	+
8	76/M	No	63/140	PD	32/35	109.4	–

†Evaluation of CT images was done by the following: PD, progressive disease; SD, stable disease. ‡CTL frequencies of pre- and post-vaccinated patients were assessed with a HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with a HLA-A24-restricted HIV peptide (RYLRDQQL) tetramer used as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTL among 104 CD8 T cells is shown. §Positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFN $\gamma$ -positive spots compared with HIV peptide-specific CD8 T cell spots, whereas negative (–) means a less than twofold increase. CEA, carcinoembryonic antigen; CT, computed tomography; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunosorbent spot; HIV, human immunodeficiency virus; IFA, incomplete Freund's adjuvant; IFN, interferon.

Consequently, it appeared that there was a close correlation between clinical SD outcomes and a reduced CEA level.

**Immune responses, single-cell cloning and subsequent functional assessment of tetramer-positive CTL.** We determined if the survivin-2B80-88 peptide vaccination could actually induce specific immune responses in the patients enrolled. The peptide-specific CTL frequency was analyzed using the HLA-A24/peptide tetramer. The CTL frequency before the first vaccination (pre-vaccination) and after the last vaccination (post-vaccination) was assessed with a HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with a HLA-A24-restricted HIV peptide (RYLRDQQL) tetramer for the negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CD8 T cells in 10<sup>4</sup> CD8 T cells was determined. In the current study, ELISPOT was also carried out using these peptides.

The data are summarized in Tables 1 and 2. In the first vaccination protocol, that is, vaccination with survivin-2B80-88 plus IFA, patients did not show obvious survivin-2B80-88-specific CTL responses in the tetramer study. Only patients Nos 2 and 3 exhibited very slight enhancement in that study. Patient No. 5 was positive in the ELISPOT study, but did not show a survivin-2B80-88-specific CTL response in the tetramer study. In these patients, there was no correlation between the clinical and immune responses. However, in the second protocol with survivin-2B80-88 plus IFA and IFN $\alpha$ , many patients demonstrated enhanced tetramer frequency. In particular, four of the eight patients (Nos 3, 5, 6 and 7) had enhanced frequency with a more than 200% increase. It was also interesting that all four of these patients were also positive in the ELISPOT study, and two had SD by CT evaluation, suggesting that immune responses might appropriately reflect, at least in some patients, clinical responses with this second vaccination protocol.

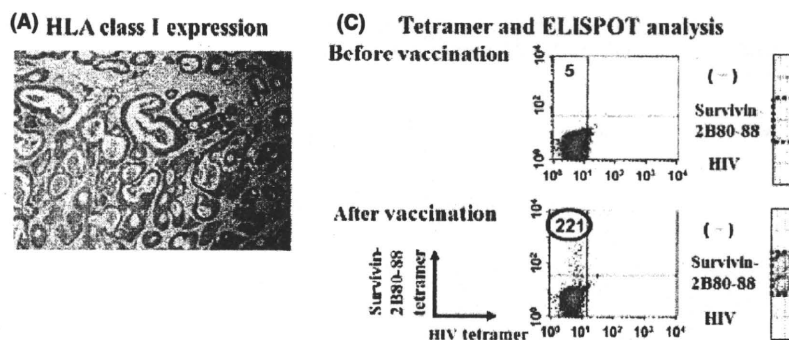
Subsequently, we analyzed tetramer-positive CD8 T cells at the single-cell level, and determined whether these T cells had specificity for the survivin-2B80-88 peptide and cytotoxic potential against live survivin-2B-positive tumor cells in the context of HLA-A\*2402. As shown in Figure 2, patient No. 6 (44 years old, female) had positive HLA class I expression in rectal tumor tissue (Fig. 2A), a reduced serum CEA level (Fig. 2B) and obvious immune responses as assessed by the survivin-2B80-88 tetramer and ELISPOT (Fig. 2C) after vaccination with the second protocol. CD8 T cells of the tetramer-positive fraction were sorted by FACS, and then cultured with 1,

3 and 10 cells/well for 7–10 days. As shown in Figure 3, almost all growing T cells were survivin-2B peptide-specific T cells, and we subsequently assessed peptide-specific cytotoxicity by using T cells of well (a) through well (h) as indicated. Consequently, as Figure 4 clearly shows, all T cells except for those in well (b) had very high peptide-specific cytotoxic potential. Furthermore, T cells in well (d) were cloned, and we obtained a T cell clone designated D-13 showing survivin-2B peptide specificity as shown in Figure 5A,B. Finally, the cytotoxic potential against survivin-2B-positive live tumor cells was assessed, confirming that the D-13 T cell clone was clearly cytotoxic against survivin-2B-positive tumor cells in a HLA-A\*2402-restricted fashion. Taken together, these data clearly indicate that the second vaccination protocol was capable of inducing a strong CTL response, and in some patients might result in clinical effectiveness.

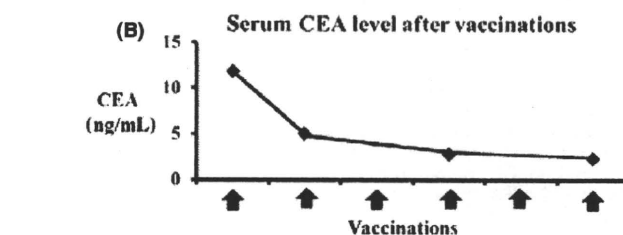
## Discussion

The immunogenicity of HLA class I-restricted tumor peptides is not strong enough for routine clinical use as cancer treatment, and it is important to increase their relative and absolute immunogenicity levels by various means. Previously, our group conducted clinical trials of the survivin-2B80-88 peptide vaccine alone and with IFA in patients with various cancers.<sup>(3,8,9,11)</sup> In the current study we determined whether the immunogenicity of survivin-2B80-88 could be enhanced with other vaccination protocols. Thus, we compared clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus IFA; and (ii) survivin-2B80-88 plus IFA and a type-I IFN, IFN $\alpha$ . Our data strongly suggest that although the effect with survivin-2B80-88 plus IFA was not different from that with the survivin-2B80-88 peptide alone, treatment with survivin-2B80-88 plus IFA and IFN $\alpha$  resulted in clear improvement of the clinical and immunological responses of patients.

There has been less evidence concerning the extent to which peptide-specific CTL responses in patients treated with peptide vaccines could occur at the single-cell level. To assess this point, CTL of patients were sorted to the single-cell level, and we confirmed that each CTL obtained from vaccinated patients was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules.



**Fig. 2.** Representative illustration of clinical effect and immunological analysis in patient No. 6 who was treated with survivin-2B80-88 plus IFA with IFN $\alpha$ . (A) Immunohistochemical analysis of HLA class I expression in primary colon cancer tissue as assessed by anti-HLA class I heavy chain monoclonal antibody EMR8-5, (B) profile of serum CEA level after vaccinations (arrows) and (C) tetramer and ELISPOT analyses before and after vaccinations. The number in the tetramer analysis indicates survivin-2B80-88 peptide-specific CD8 $^{+}$  T cells among 10 $^4$  CD8 $^{+}$  T cells. CEA, carcinoembryonic antigen; ELISPOT, enzyme-linked immunosorbent spot; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; IFA, incomplete Freund's adjuvant; IFN, interferon.



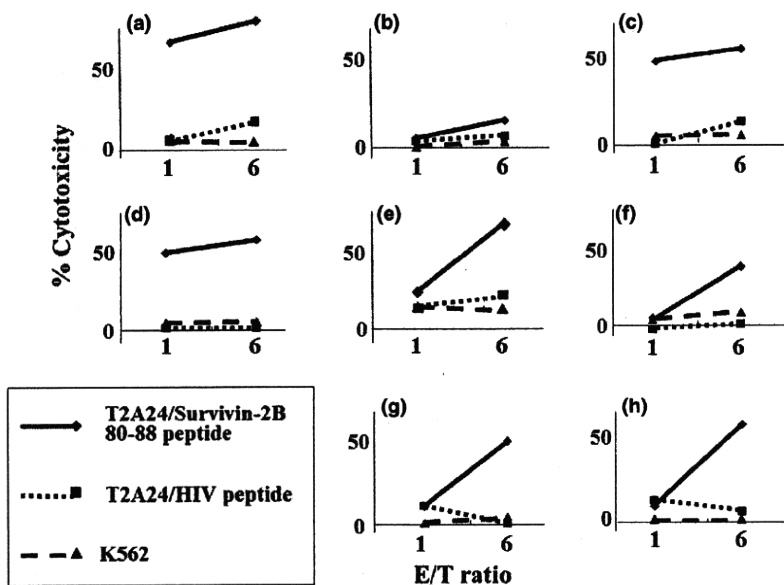
**Fig. 3.** Single-cell analysis of survivin-2B80-88 peptide tetramer-positive CD8 T cells. Survivin-2B80-88 peptide tetramer-positive CD8 T cells in Figure 2C (circle) were sorted and cultured at 10 (A), 3 (B) and 1 (C) cells/well for 7–10 days. Subsequently, cells were examined for their reactivity to the survivin-2B80-88 peptide tetramer.

It is well known that type-I interferons such as IFN $\alpha$  can work in many immunological manners to activate T cell responses.<sup>(22–25)</sup> The maturation of dendritic cells (DC) and their effect on the expression of HLA molecules seems to be the main action of this cytokine. Because of difficulties with the availability of patients' samples, we could not actually compare these features of patients' DC and primary tumor tissues before and after treatment with IFN $\alpha$ . However, the evidence of obvious enhancement of CTL responses and improvement of clinical responses in our study favors the two main actions described above. Indeed, a preliminary study of three colon patients who received survivin-2B80-88 plus IFA with GM-CSF did not demonstrate any clinical or immunological response, suggesting that the action of IFN $\alpha$  is remarkable from the aspect of being an immunogenic enhancer for human peptide vaccines. Ideally, patients should be administered IFN $\alpha$  alone before vaccination treatment is undertaken. However, this was practically hard to do since all patients were in the

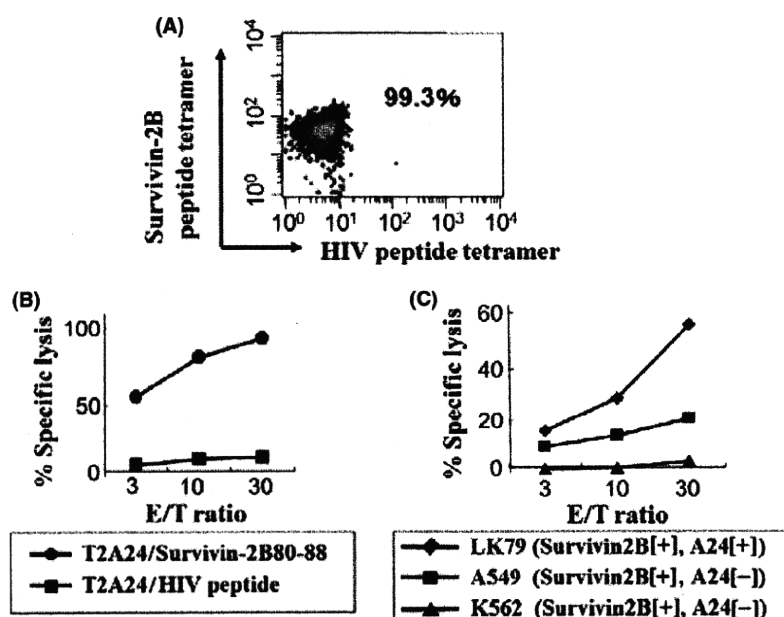
late and advanced disease stages and it was difficult to select such patients. Meanwhile, there is no evidence that administration of IFN $\alpha$  alone results in overt clinical benefits such as those observed in the current study. The second vaccination protocol using IFN $\alpha$  appeared to be highly efficient for enhancing peptide immunogenicity.

Nevertheless, we require more efficient and reasonable ways to enhance peptide immunogenicity *in vivo* with limited side-effects.<sup>(26)</sup> In our laboratory, it was observed that heat shock protein 90 (HSP90) could highly selectively target substrates such as HLA-class I restricted peptides as well as the innate immunity ligand, CpG, to the Rab5 (+) and EEA1 (+) early static endosomes of DC.<sup>(3,27–29)</sup> Consequently, HSP90 could strongly induce peptide-specific CTL responses *in vitro* and *in vivo* without obvious side-effects,<sup>(20)</sup> and it is intriguing that its action may surpass the benefits of IFN $\alpha$ .

Collectively, our present study may reflect one potential vaccination protocol. Since the vaccination protocol with



**Fig. 4.** Analysis of survivin-2B80-88 peptide-specific cytotoxicity. T cells of wells (a) through (h) in Figure 3 were assessed for their peptide-specific cytotoxicity against T2A24 target cells pulsed with the survivin-2B80-88 peptide and HIV peptide, and against control K562 cells. E/T, effector/target; HIV, human immunodeficiency virus.



**Fig. 5.** Clonal analysis of survivin-2B80-88 peptide-specific CD8 T cells. T cells in well (d) in Figure 3 were cultured and CTL clone D-13 was obtained. Subsequently, D-13 was studied for (A) tetramers and (B) cytotoxicity against T2A24 cells pulsed with survivin-2B80-88 and HIV peptides, and (C) cytotoxicity against human tumor cells (LK79, A549 and K562) that express survivin and/or HLA-A24 molecules as indicated. CTL, cytotoxic T lymphocyte; E/T, effector/target; HIV, human immunodeficiency virus; HLA, human leukocyte antigen.

survivin-2B80-88 plus IFA and IFN $\alpha$  appears effective in other cancers such as pancreatic cancer as well, this protocol may be useful as a standard immunotherapy modality.

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### Disclosure Statement

The authors have no conflict of interest.

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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).<sup>[1–8]</sup> We have launched clinical trials of cancer vaccine therapy with HLA-A24–restricted antigenic peptides.<sup>[9,10]</sup> 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.<sup>[11,12]</sup> 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<sup>[13,14]</sup>; 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  $\mu$ L 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  $\mu$ L of PCR mixture containing 1  $\mu$ L of genomic DNA, 0.1  $\mu$ L 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'-ACTGACCGAGAGAACCTGCCGAT-3' and 5'-ACTTGGCGCTTGGTGATCTGAGCC-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'-ACTTGGCGCTTGGTGATCTGAGCC-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).

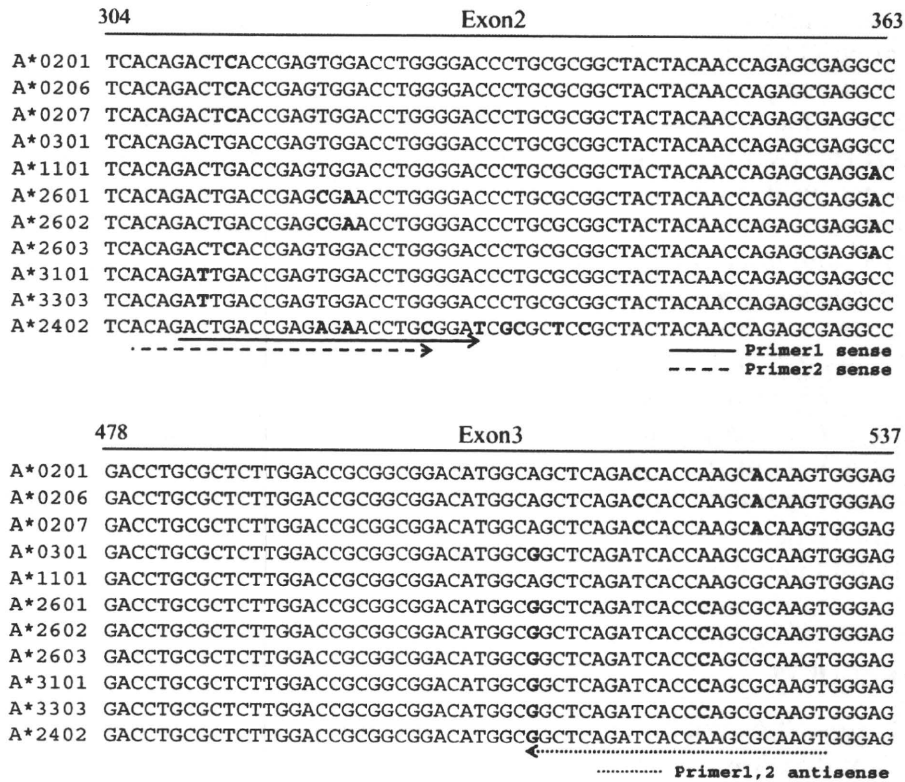
## RESULTS

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

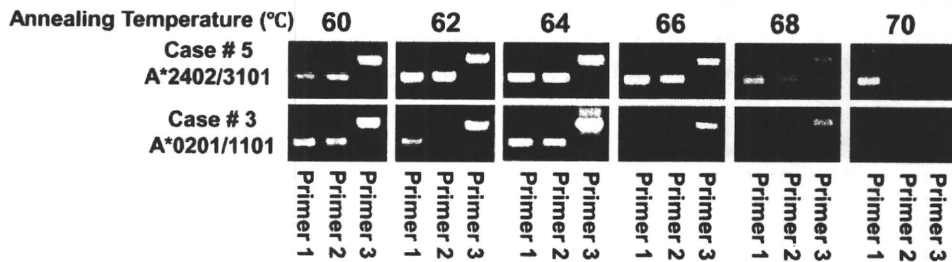
To establish the one-step PCR-SSP method, we designed two novel HLA-A24-specific primer sets 23 bps in length (Table 1 and Figure 1). To determine the appropriate annealing temperature, we performed PCR amplification with serial annealing temperatures to amplify the HLA-A\*2402-positive and HLA-A\*2402-negative 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	ACTGACCGAGAGAACCTGCCGAT
	antisense	ACTTGGCGCTTGGTGATCTGAGCC
Primer 2	sense	ACAGACTGACCGAGAGAACC TGC
	antisense	ACTTGGCGCTTGGTGATCTGAGCC
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.

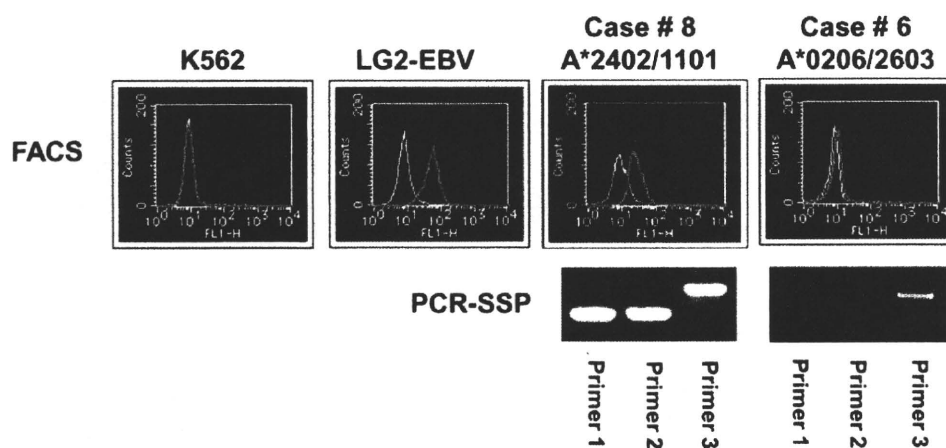


70°C. The PCR bands were confirmed to be identical with HLA-A\*2402 sequence by DNA direct sequencing (data not shown). Thus, HLA-A24-specific amplification could be attained with annealing temperatures of 68°C and 70°C. Therefore, we have chosen 68°C as the annealing temperature for HLA-A24-specific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

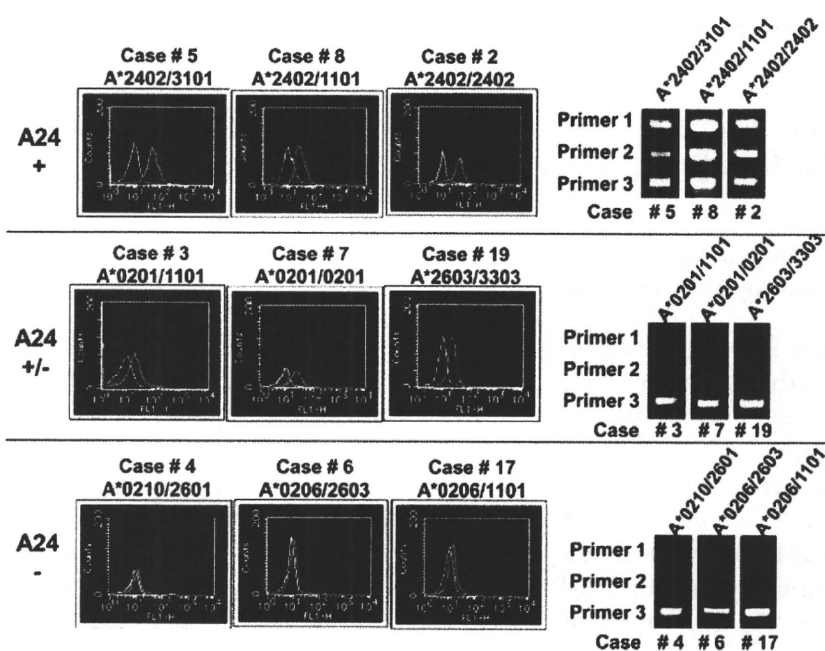
### Detection of HLA-A24 with the PCR-SSP and Serological Methods

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.<sup>[15]</sup> 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.<sup>[11,12]</sup> There are already several reports of methods for DNA typing of specific alleles.<sup>[16-19]</sup> 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 exon 3. Exon 2 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.<sup>[20]</sup> Laforet et al. showed that an intronic mutation caused lower expression of the HLA-A24 allele.<sup>[21]</sup> 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.

## ACKNOWLEDGMENTS

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*Stem Cells, Tissue Engineering, and Hematopoietic Elements*

## Cytotoxic T Lymphocytes Efficiently Recognize Human Colon Cancer Stem-Like Cells

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**Cancer stem-like cells (CSCs) and tumor-initiating cells (TICs) are a small population of cancer cells that share three properties: tumor initiating ability, self-renewal, and differentiation. These properties suggest that CSCs/TICs are essential for tumor maintenance, recurrence, and distant metastasis. Here, we show that cytotoxic T lymphocytes (CTLs) specific for the tumor-associated antigen CEP55 can efficiently recognize colon CSCs/TICs both *in vitro* and *in vivo*. Using Hoechst 33342 dye staining, we isolated CSCs/TICs as side population (SP) cells from colon cancer cell lines SW480, HT29, and HCT15. The SP cells expressed high levels of the stem cell markers SOX2, POU5F1, LGR5, and ALDH1A1 and showed resistance to chemotherapeutic agents such as irinotecan or etoposide. To evaluate the susceptibility of SP cells to CTLs, we used CTL clone 41, which is specific for the CEP55-derived antigenic peptide Ccp55/c10orf3\_193 (10) (VYVKGLLAKI). The SP cells expressed HLA class I and CEP55 at the same level as the main population cells. The SP cells were susceptible to CTL clone 41 at the same level as main population cells. Furthermore, adoptive transfer of CTL clone 41 inhibited tumor growth of SW480 SP cells *in vivo*. These observations suggest that Ccp55/c10orf3\_193(10) peptide-based cancer vaccine therapy or adoptive cell transfer of the CTL clone is a possible approach for targeting chemotherapy-resistant colon CSCs/TICs. (Am J Pathol 2011, 178:1805–1813; DOI: 10.1016/j.ajpath.2011.01.004)**

Colon cancer is one of the most common malignancies worldwide. With recent progress in treatment, the prognosis has improved to some extent. In advanced disease, however, the prognosis remains unfavorable, because of recurrence, distant metastasis, and resistance to treatment. Thus, novel treatment modalities are needed.

Cancers contain morphologically heterogeneous populations. This fact has led to the cancer stem cell theory,<sup>1</sup> the idea that cancers are composed of several types of cells, and that only a small population of cancer cells that can regenerate cancer tissues, much as normal tissue can be regenerated only by a small population of stem-like cells. Recently, cancer stem-like cells and tumor-initiating cells (CSCs/TICs) have been isolated from various types of malignancies, including colon cancer.<sup>2–6</sup> In colon cancer, CSCs/TICs can reinitiate tumors that resemble mother colon cancer tissues morphologically when transplanted into immunodeficient mice.<sup>3</sup> Furthermore, these CSCs/TICs have higher tumorigenic potential than do non-CSCs/TICs. Previous reports have shown that CSCs/TICs are resistant to a variety of treatments, including chemotherapy and radiotherapy, with varied mechanisms of resistance, including high expression of drug transporters, relative cell cycle quiescence, high levels of DNA repair machinery, and resistance to apoptosis.<sup>7</sup> These reports<sup>3–6</sup> support the hypothesis that malignant cancers comprise heterogeneous populations that organize in a hierarchical differentiation model. The CSCs/TICs are located at the top of this hierarchy, and targeting CSCs/TICs is essential to achieve efficient effects for treatment of malignant diseases. Recently, some trials targeting CSCs/TICs have been reported for hema-

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topoietic malignancies.<sup>8</sup> Hedgehog signaling is essential for maintenance of myeloid leukemia stem cells, and inhibition of hedgehog signaling by cyclopamine is effective for imatinib-resistant myeloid leukemia.<sup>9</sup> To date, however, no such CSC/TIC targeting approach has been reported for colon cancer.

In the present study, we evaluated the efficiency of CTL-based immunotherapy targeting colon CSCs/TICs. Using Hoechst 33342 dye, we isolated colon CSCs/TICs as side population (SP) cells from six colon cancer cell lines. The SP cells derived from SW480, HT29, and HCT15 showed higher tumorigenicity than did main population (MP) cells. On the other hand, SP cells from KM12LM, Lovo, and Colo320 did not show any increase in tumorigenicity, compared with MP cells. This suggests that SW480, HT29, and HCT15 SP cells (but not KM12LM, Lovo, and Colo320 SP cells) were enriched with CSCs/TICs. In RT-PCR analysis the SW480, HT29, and HCT15 SP cells showed a stem cell-like gene expression signature, including SOX2, POU5F1, LGR5, and ALDH1A1. Furthermore, these SP cells also showed resistance to chemotherapeutic agents, including irinotecan and etoposide. These observations support the idea that these SP cells had stem cell-like features. To assess the immunogenicity of SP cells, we evaluated the expression of HLA class I and of CEP55, which is a tumor-rejection antigen of breast and colon cancer.<sup>10,11</sup> The SP cells expressed HLA class I (and also HLA-A24) at the same level as MP cells. The SP cells also expressed CEP55 messenger RNA (mRNA) at the same level as MP cells in RT-PCR. To confirm the susceptibility of SP cells to cytotoxic T lymphocytes (CTLs), we used CTL clone 41, which recognizes CEP55 in an HLA-A24-restricted manner.<sup>10</sup> CTL clone 41 killed SW480, HT29, and HCT15 SP cells at the same level as it killed MP cells and presorted cells. These observations suggest that colon CSCs/TICs are also sensitive to CTLs, as non-CSC/TIC populations are. Furthermore, adoptive transfer of CTL clone 41 inhibited the tumor growth of SW480 SP cells in immunodeficient mice. These observations suggest that CTL-based colon cancer immunotherapy is efficient for colon CSCs/TICs. To our knowledge, the present study provides the first direct evidence that colon CSCs/TICs are susceptible to CTLs and thus opens possibilities for future applications in immunotherapy using CSC/TIC-specific vaccines.

## Materials and Methods

### Cell Lines

Colon adenocarcinoma cell lines SW480 (HLA-A\*0201/2402), HCT15 (HLA-A\*0201/2402), HT29 (HLA-A1/24), Lovo, and Colo320 were kind gifts of Dr. K. Imai (Sapporo, Japan), and KM12LM was a kind gift of Dr. K. Itoh (Kurume, Japan). All cell lines except K562 were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). K562 was cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal

bovine serum. HCT15-B2M, a stable transfectant of HCT15 cells with B2M ( $\beta 2$  microglobulin) cDNA, was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10  $\mu\text{g}/\text{mL}$  puromycin (Sigma-Aldrich).<sup>11</sup>

### Side Population Analysis

Side population analysis was performed as described previously, with some modifications.<sup>12</sup> Trypsinized cultured cells were washed with PBS and were resuspended at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. After 10 minutes preincubation, the cells were labeled with Hoechst 33342 dye (Lonza, Walkersville, MD) for 90 minutes at concentrations of 3.75  $\mu\text{g}/\text{mL}$  for Colo320, 5  $\mu\text{g}/\text{mL}$  for SW480 and Lovo, 7.5  $\mu\text{g}/\text{mL}$  for HT29 and KM12LM, and 10  $\mu\text{g}/\text{mL}$  for HCT15, with or without verapamil (Sigma-Aldrich), which is an inhibitor of ABC transporters, at concentrations of 50  $\mu\text{mol}/\text{L}$  for SW480, HCT15, and Colo320, 75  $\mu\text{mol}/\text{L}$  for Lovo, and 100  $\mu\text{mol}/\text{L}$  for HT29. Cells were counterstained with 1  $\mu\text{g}/\text{mL}$  propidium iodide to label dead cells. Next,  $1 \times 10^6$  viable cells were analyzed and sorted using a BD FACSAria II fluorescence-activated cell sorting system (BD Biosciences, Franklin Lakes, NJ). The Hoechst dye was excited at 355 nm, and its fluorescence was measured at two wavelengths using optical filters 405 DF20 [450/20 nm band-pass filter O (Hoechst Blue)] and 635LP [635 nm long-pass edge filter (Hoechst Red)]. Propidium iodide labeling was measured through a 630/BP30 filter for discrimination of dead cells.

### Xenograft Model

The SP cells, MP cells, and presorted cells from colon cancer cell lines were mixed 1:1 by volume with Matrigel (BD Biosciences) and were injected subcutaneously into the backs of female 4- to 8-week-old nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Tumor size in cubic millimeters was assessed weekly with calipers and was calculated as Tumor Size = (Longest Diameter  $\times$  Shortest Diameter<sup>2</sup>)/2.

### RT-PCR Analysis of SP and MP Cells

RT-PCR analysis was performed as described previously.<sup>10</sup> Total RNAs were isolated from both SP cells and MP cells using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 2  $\mu\text{g}$  of total RNA by reverse transcription using SuperScript III reverse transcriptase (Invitrogen). The PCR amplification was performed in 20  $\mu\text{L}$  of PCR mixture containing 1  $\mu\text{L}$  of cDNA mixture, 0.5  $\mu\text{L}$  of Taq DNA polymerase (Qiagen) and 4 pmol of primers. The PCR mixture was initially incubated at 98°C for 2 minutes, followed by 30 cycles of denaturation at 98°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The following primer pairs were used for RT-PCR analysis (forward and reverse, respectively): 5'-CATGATG-

GAGACGGAGCTGA-3' and 5'-ACCCCGCTCGCCATGCTATT-3' for SOX2, with an expected PCR product size of 410 bp; 5'-TGGAGAAGGAGAAGCTGGAGCAAAA-3' and 5'-GGCAGATGGTCGTTGGCTGAATA-3' for POU5F1, with an expected PCR product size of 163 bp; 5'-CTCTT CCTCAAACCGTCTGC-3' and 5'-GATCGGAGGCTAAGCAACTG-3' for LGR5, with an expected PCR product size of 181 bp; 5'-TGTTAGCTGATGCCGACTTG-3' and 5'-TTCTTAGCCCGCTCAACACT-3' for ALDH1A1, with an expected PCR product size of 154 bp; 5'-TGAGTTT-GCCATCACAGAGC-3' and 5'-TTGCTTGCTGGTGCATTAAC-3' for CEP55, with an expected PCR product size of 521 bp; and 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with an expected product size of 452 bp. GAPDH was used as an internal control.

### Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Primers and probes were designed by the manufacturer (TaqMan gene expression assays; Applied Biosystems). Thermal cycling was performed using 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Each experiment was done in triplicate, with normalization to the *GAPDH* gene as an internal control.

### Flow Cytometric Analysis and Monoclonal Antibodies

Cells were incubated with mouse monoclonal antibodies at saturation concentration for 30 minutes on ice, washed with PBS, and stained with a polyclonal goat anti-mouse antibody coupled with fluorescein isothiocyanate for 30 minutes. Samples were analyzed using a BD FACSCalibur flow cytometry system (Becton Dickinson, Mountain View, CA). Anti-pan HLA class I (W6/32) and anti-HLA-A24 monoclonal antibodies (C7709A2.6 hybridoma, a kind gift from Dr. P.G. Coulie, Brussels, Belgium) were prepared from hybridomas.

### Survival Studies for Etoposide and Irinotecan

We isolated SP and MP cells of SW480 and HCT15 and seeded them into 96-well culture plates at  $1 \times 10^4$  cells per well for each population of cells. The cells in both populations were treated with etoposide (1 and 5  $\mu\text{g}/\text{mL}$ ) or irinotecan (40 and 400  $\mu\text{g}/\text{mL}$  for SW480, 10 and 100  $\mu\text{g}/\text{mL}$  for HCT15). After 72 hours of exposure to the chemotherapeutic agents, viability of the cells was determined using the SOD assay kit WST-1, which was performed according to the manufacturer's protocol (Dojindo Molecular Technologies, Kumamoto, Japan; Rockville, MD).

### Cytotoxicity Assay for SP Cells with CTL Clone 41

We had previously established CTL clone 41, which recognizes an HLA-A24 restricted antigenic peptide (VYVK-GLLAKI) termed Cep55/c10orf3\_193(10), from an HLA-A24-positive breast cancer patient's peripheral blood mononuclear cells.<sup>8</sup> The lytic activity of CTL clone 41 for SP cells, MP cells, and presorted cells was evaluated by <sup>51</sup>Cr release assay. Briefly, SP cells, MP cells and presorted cells were labeled with 100  $\mu\text{Ci}$  of <sup>51</sup>Cr for 1 hour at 37°C, washed four times with PBS, and resuspended in AIM-V medium (Invitrogen). The <sup>51</sup>Cr-labeled target cells (2000 cells/well) were then incubated with various numbers of effector cells for 6 hours at 37°C in 96-well culture plates. Radioactivity of the culture supernatant was measured with a gamma counter. The percentage of cytotoxicity was calculated as follows: % Specific Lysis = (Experimental Release – Spontaneous Release)  $\times$  100 / (Maximum Release – Spontaneous Release). Target cells were treated with 100 units/mL interferon- $\gamma$  for 48 hours before the assay.

### Winn Assay

SW480 SP cells were mixed with CTL clone 41 at a ratio of 1 SP cell to 10 CTL cells. The resulting mixture (200  $\mu\text{L}$  with  $1 \times 10^6$  CTL clone 41 and  $1 \times 10^5$  SP cells) was injected subcutaneously into the backs of NOD/SCID mice. A control group of five mice was injected with SP cells alone. Tumor size was assessed weekly.

### CTL Adoptive Transfer

NOD/SCID mice were inoculated subcutaneously on the back with  $1 \times 10^3$  SW480 SP cells. Three weeks later, when the tumor started to be palpable,  $5 \times 10^4$  Cep55/c10orf3\_193(10)-specific CTL clone cells or PBS was injected intravenously. The same adoptive transfer procedure was performed 4 weeks after inoculation with SP cells. Tumor size was assessed weekly.

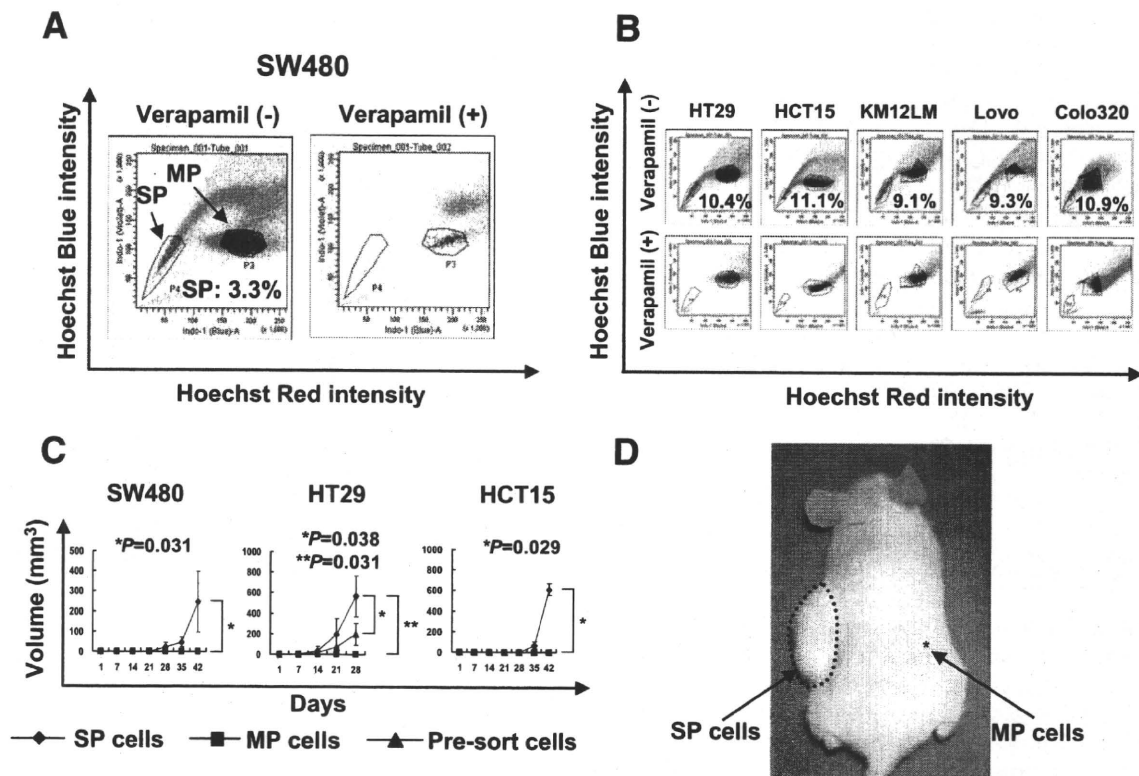
### Statistical Analysis

In the xenograft model, survival studies using chemotherapeutic agents, cytotoxicity assay, Winn assay, and adoptive transfer model, the data were analyzed using the Mann-Whitney *U*-test, with  $P < 0.05$  conferring statistical significance.

## Results

### Isolation of Colon CSCs/TICs as SP Cells

Several methods to isolate colon cancer CSCs/TICs has been reported, including cell surface markers such as CD44 or PROM1 (CD133), SP cells, and the Aldefluor assay.<sup>3-6,13</sup> In the present study, we isolated colon CSCs/TICs using SP cell analysis. Several colon cancer cell lines were dyed with Hoechst 33342 and then analyzed with a BD FACSAria II flow cytometer as



**Figure 1.** Isolation of colon CSCs/TICs from colon cancer cell lines and tumor growth of the SP cells. **A:** Colon cancer cell line SW480 was stained with Hoechst 33342 dye as described under *Materials and Methods*, with or without verapamil. Stained cells were analyzed using a BD FACSAria II fluorescence-activated cell sorting system. Frequency of SP cells was 3.3%. **B:** Colon cancer cell lines (HT29, HCT15, KM12LM, Lovo, and Colo320) were stained with Hoechst 33342 dye with or without verapamil. Stained cells were analyzed using a BD FACSAria II system. Frequencies of SP cells ranged from 9.1% for KM12LM cells to 11.1% for HCT15 cells. **C:** SP cells, MP cells, and presorted cells of colon cancer cell lines SW480, HT29, and HCT15 were inoculated subcutaneously into the backs of NOD/SCID mice ( $1 \times 10^3$  cells injected). Data are reported as means  $\pm$  SD. *P* values indicate differences between cell types according to a Mann-Whitney *U*-test. **D:** Representative tumor growth in NOD/SCID mice at the SP cell injection site ( $1 \times 10^3$  cells injected). SP cells and MP cells were inoculated subcutaneously into the left and right side of the back, respectively.

described under *Materials and Methods*. Side population cells could be detected in all six colon cancer cell lines analyzed (ie, SW480, HT29, HCT15, Colo320, Lovo, and KM12LM) (Figure 1, A and B). The frequency of SP cells ranged from 3.3% for SW480 to 11.1% for HCT15 cells. All these SP cells were specifically inhibited by verapamil, as has been shown previously,<sup>14</sup> suggesting that these SP cells were specific for ABC transporter expression. Because previous studies showed that some colon cancer SP cells were not enriched with a CSC/TIC population,<sup>15</sup> it was essential to confirm the presence of CSCs/TICs in SP cells for further analysis. We inoculated these SP cells subcutaneously into the back of immunodeficient NOD/SCID mice using serial dilution. The SP cells derived from SW480, HCT15, and HT29 showed higher tumor initiating ability, compared with MP cells (Table 1). Furthermore, SW480, HT29, and HCT15 SP cells showed faster tumor growth, compared with MP cells (Figure 1, C and D), suggesting the presence of CSCs/TICs in these SP cells. In contrast, the SP cells derived from Colo320, Lovo, and KM12LM did not show any difference in tumorigenicity or tumor growth, compared with MP cells. We therefore restricted further analysis to the SW480, HT29, and HCT15 SP cells as colon cancer CSCs/TICs.

#### RT-PCR Analysis of Colon Cancer SP Cells

To examine the molecular properties of SP cells, we performed RT-PCR analysis. SOX2 and POU5F1 are representative markers for embryonal stem cells and CSCs/TICs.<sup>16</sup> The SP cells derived from SW480, HT29, and HCT15 showed higher expression of both SOX2 and POU5F1, compared with MP cells (Figure 2A). ALDH1A1, a colon CSC/TIC marker,<sup>6</sup> was expressed at a higher level in SP cells of HCT15 than in MP cells, but SP cells of SW480 and HT29 did not show any difference in comparison with MP cells. SW480 and HT29 SP cells also showed higher expression of LGR5, which is known as a normal colon stem cell marker.<sup>17</sup> To confirm the expression of stem cell markers, we also performed real-time PCR. The SW480 SP cells expressed 90 times higher SOX2, 7 times higher POU5F1, 153 times higher LGR5, and 6.1 times higher ALDH1A1, compared with MP cells (Figure 2B). These findings indicate that these SP cells had molecular properties similar to those of embryonal stem cells.

#### Resistance to Chemotherapeutic Reagents

Although SP cells derived from liver cancer cell line HuH7 have showed resistance to chemotherapy,<sup>13</sup> we know of no conclusive previous studies of such resistance in co-



**Table 1.** Tumor Initiating Ability of Colon Cancer SP Cells

Cell line (% SP cells)	Tumor initiating ability*		
	1 × 10 <sup>4†</sup>	1 × 10 <sup>3†</sup>	1 × 10 <sup>2†</sup>
SW480 (3.3)			
SP cells	4/4	4/6	4/4
MP cells	2/4	3/5	0/4
HT29 (10.4)			
SP cells	3/3	2/3	3/3
MP cells	3/3	0/3	0/3
HCT15 (11.1)			
SP cells	3/3	3/4	3/3
MP cells	1/3	1/4	0/3
Colo320 (10.9)			
SP cells	2/2	1/2	1/2
MP cells	2/2	2/2	1/2
Lovo (9.3)			
SP cells	0/1	1/1	0/1
MP cells	1/1	0/1	0/1
KM12LM (9.1)			
SP cells	1/2	2/2	1/1
MP cells	1/2	2/2	1/1

MP, main population; SP, side population.

\*Tumor initiating ability is expressed as the ratio of tumor-initiation to injection.

†The tumor initiation abilities were evaluated at day 42 after injection of the indicated number of cells.

ion SP cells. We performed a cell survival study of colon cancer SP cells using the chemotherapeutic agents irinotecan and etoposide. The SW480 and HCT15 SP cells were more resistant to both irinotecan and etoposide than were MP cells (Figure 3, A and B). This finding is consistent with findings for CSCs/TICs derived from other organs.<sup>22,24</sup>

#### Expression of HLA and Tumor-Associated Antigens in SP Cells

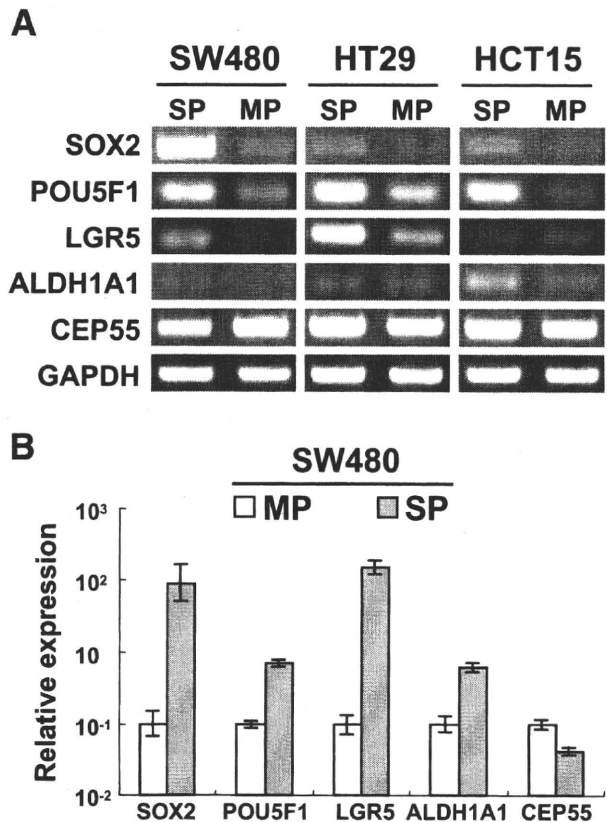
Because CTLs recognize tumor-associated antigen (TAA)-derived antigenic peptides presented by HLA class I molecules, expression of HLA class I molecules is essential for activation of CTLs. Several types of malignancies have been reported to lose the expression of HLA class I molecules through various mechanisms and so escape CTL attack.<sup>19</sup> We therefore evaluated the expression of HLA class I and HLA-A24 expression between SP cells and MP cells by flow cytometry. Because ELISA study has revealed that HCT15 cells lack B2M because of gene mutations of *B2M*,<sup>19</sup> we transduced wild-type B2M cDNA into HCT15 cells and so established HCT15-B2M cells. The SW480, HT29, and HCT15-B2M SP cells showed HLA class I and HLA-A24 expression at the same level as MP cells (Figure 4, A and B). Furthermore, we assessed the expression of one of the colon cancer TAAs, CEP55, by both RT-PCR and real-time PCR (Figure 2, A and B). Both SP cells and MP cells derived from SW480, HT29, and HCT15-B2M expressed CEP55 mRNA at the same level. These data raised the possibility that SP cells are also sensitive to CTLs specific for the CEP55-derived antigenic peptide. Because both SP cells and MP cells expressed CEP55 mRNA at the same level, this appeared

to be an ideal target for comparing the susceptibilities of SP cells and MP cells to CTLs.

#### Susceptibility of CSCs/TICs to CTLs, in Vitro and in Vivo

We had previously established CTL clone 41, which is specific for the cancer-related, antigen-derived, HLA-A24-restricted peptide Cep55/c10orf3\_193(10).<sup>10</sup> CTL clone 41 recognized CEP55-positive and HLA-A24-positive cancer cells, as described previously.<sup>10,11</sup> In the present study, we used a <sup>51</sup>Cr release assay to examine whether CTL clone 41 can recognize SP cells. All SP cells derived from SW480, HT29, and HCT15-B2M showed susceptibility to CTL clone 41 at the same level as the MP cells and the presorted bulk cell lines (Figure 4C). This indicates that the colon CSCs/TICs were sensitive to CTLs.

To analyze the cytotoxic activity of the CTL clone *in vivo*, we performed a Winn assay. SW480 SP cells with or without CTL clone 41 were injected into the backs of NOD/SCID mice subcutaneously. CTL clone 41 significantly inhibited the tumorigenicity of SW480 SP cells (Figure 4D). Because CTLs were injected at the same time and place as the SP cells in the Winn assay, we could not rule out the possibility that SP cells were killed *in vitro*. We



**Figure 2.** Expression of stem cell markers in SP and MP cells. **A:** mRNAs purified from SW480, HT29, and HCT15 SP and MP cells were analyzed by RT-PCR. **B:** mRNA purified from SW480 SP cells and MP cells were analyzed with real-time PCR. mRNA expression level is relative to MP cells. Data are reported as means ± SD.