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Implication of chemo-resistant memory T cells for immune surveillance in patients with sarcoma receiving chemotherapy

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Chemotherapy has improved the prognosis of patients with sarcomas. However, it may suppress anti-tumor immunity. Recently, we reported a novel CD8$^+$ memory T cell population with a chemo-resistance property, "young memory" T (T_{YM}) cells. In this study, we investigated the proportion and function of T_{YM} cells in peripheral blood of healthy donors and sarcoma patients who received chemotherapy and those who did not. The proportion of T_{YM} cells was significantly decreased in patients compared with that in healthy donors. In healthy donors, anti-EBV CTLs were induced using mixed lymphocyte peptide culture, from not only T_{YM} cells but also T_{CM} and T_{EM} cells. No CTLs directed to tumor-associated antigens were induced. In sarcoma patients who did not receive chemotherapy, in addition to anti-EBV CTLs, CTLs directed to the tumor-associated antigen PBF were induced from T_{YM}, T_{CM} and T_{EM} cells. In sarcoma patients who received chemotherapy, EBV-specific CTLs were induced from T_{YM} cells but were hardly induced from T_{EM} cells. Interestingly, CTLs directed to the anti-tumor-associated antigen PBF were induced from T_{YM} cells but not from the T_{CM} and T_{EM} cells in sarcoma patients who received chemotherapy. The findings suggest that T_{YM} cells are resistant to chemotherapy and can firstly recover from the nadir. T_{YM} cells might be important for immunological memory, especially in sarcoma patients receiving chemotherapy.

Bone and soft tissue sarcomas, especially osteosarcoma, are highly malignant neoplasms and that occurred in children and young adults. The introduction of high-dose chemotherapy has increased the 5-year overall survival of patients with osteosarcoma from 10% up to 70%.[1] However, the prognosis of non-responders to chemotherapy is still poor and new therapeutic modalities are required. We have focused on the development of immunotherapy for sarcoma. We have searched for tumor-associated antigens and CTL epitopes in the context of HLA class I, and we have performed clinical peptide vaccination for patients with osteosarcoma and synovial sarcoma targeting PBF and SYT-SSX, respectively. Immune responses were elicited in many patients, but the responses were generally weak and the objective responses were poor. Some patients showed the clinical responses, and the characteristics of those patients were that: (i) the target lesion was small (≤2 cm); and (ii) they did not receive chemotherapy.[2] These characteristics suggest that chemotherapy can kill sarcoma cells but simultaneously weakens immune surveillance and that intensive chemotherapy has poor compatibility with immunotherapy.

Recently, the existence of chemo-resistant memory T cells, now called memory T stem cells, has been reported.[3,4] At first, all T cells in neonatal infants are naive. After exposure to various pathogens, the naive T cells (T_N) differentiated into central memory (T_{CM}), effector memory (T_{EM}) and effector (T_{EFF}) T cells in the context of expression of CD45RA, CD45RO, CD62L and CCR7.[5] In the lineage of T cell differentiation, stem cell memory T cells (T_{SCM}), defined by CD95$^+$, exist between T_N and T_{CM}, and have the characteristics of chemo-resistance, long-living and differentiation into other memory T cell subsets. Recently, Murata *et al.*[6] reported a novel stem-like memory T cell population, "young memory (T_{YM})". T_{YM} cells were defined by the expression of CD73$^+$CD45RA$^+$CD62L$^+$CCR7$^+$CXCR3$^+$ and CD95$^-$. T_{YM} cells memorized virus antigens and some tumor-associated antigens in healthy donors and cancer patients, respectively. However, the alteration in the proportion of and the role of T_{YM} cells in sarcoma patients before and after chemotherapy is still unclear.

The purpose of this study was to investigate the proportions of T_{YM} cells and other CD8$^+$ T-cell subsets in healthy donors and in sarcoma patients who received or did not receive chemotherapy. We also assessed the immunological memory directed to viral antigens and tumor-associated antigens in T_{YM} cells and other T cell subsets in sarcoma patients by

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in vitro stimulation with CTL epitopes in the context of HLA-A24.

Materials and Methods

The present study was performed in accordance with the guidelines established by the Declaration of Helsinki and was approved by the Ethics Committee of Sapporo Medical University. The patients, their families, and healthy donors provided informed consent for the use of blood samples in our research.

Study participants. We obtained peripheral blood mononuclear cells (PBMCs) from 27 sarcoma patients at Sapporo Medical University, Japan. Six patients had osteosarcoma, four had chondrosarcoma, three had MPNST, three had undifferentiated pleomorphic sarcoma, three had leiomyosarcoma, two had parosteal osteosarcoma, two had myxofibrosarcoma, and one patients each had periosteal osteosarcoma, synovial sarcoma, Ewing sarcoma and epithelioid sarcoma. PBMCs were also obtained from of 23 healthy donors.

Antibodies, flow cytometry and cell sorting. Peripheral blood mononuclear cells were stained and separated into T cell subsets as previously described.[6] Briefly, PBMCs were washed twice in PBS and labeled with the following fluorescent antibodies: APC-H7-conjugated anti-CD3, FITC-conjugated anti-CD8, PE-Cy7-conjugated anti-CD45RA, APC-conjugated anti-CD62L, BV421-conjugated anti-CD73, PE-conjugated anti-CXCR3 and PerCP-Cy5.5-conjugated anti-CD95 (BD Biosciences, San Diego, CA, USA; Table S1). After incubation for 30 min at room temperature, labeled cells were analyzed using FACSAria II BD (BD Bioscience). Subsequently, $CD8^+CD73^+CD45RA^+$ $CD62L^+CXCR3^-CD95^-$ cells as the naive T cells (T_N cells), $CD8^+CD73^+CD45RA^+$ $CD62L^+CXCR3^+$ $CD95^-$ cells as the young memory T cells (T_{YM} cells), $CD8^+CD45RA^+CD62L^+$ $CXCR3^+$ $CD95^+$ cells as stem cell memory T cells (T_{SCM} cells), $CD8^+CD45RA^-CD62L^+$ cells as T_{CM} cells and $CD8^+CD45RA^-CD62L^-$ cells as T_{EM} cells were sorted. Collected data were analyzed with BD FACSDiva V6.1.3 (BD Bioscience) and GraphPad Prism software version 7 (MDF, Tokyo, Japan). The gating strategy is depicted in Figure S1.

Mixed lymphocyte peptide culture for antigen-specific CTL induction. Peripheral blood mononuclear cells obtained from HLA-A*24:02$^+$ sarcoma patients and healthy donors sorted into $CD8^+$ T-cell subsets as described above were used as responder cells. The other $CD8^-$ T cells were used as stimulator cells. $CD8^-$ cells ($1–2 \times 10^5$/well) were incubated for 90 min at room temperature with peptide mix at the concentration of 10 μg/mL. The peptides PBF A24.2 (AYRPVSRNI),[7] survivin2B (AYACNTSTL),[8] HIV env gp160 (RYLRDQQLL) and Epstein–Barr virus (EBV) BRLF1 (TYPVLEEMF) were mixed and pulsed. After incubation, responder cells ($0.5–1 \times 10^5$ well) and stimulator cells ($1–2 \times 10^5$/well) were co-cultured in 96-microwell plates in 300 μL of AIM-V (Life Technologies Japan Ltd., Tokyo, Japan) with 10% human serum (HS), IL-2 (20 IU/mL; a kind gift from Takeda Chemical Industries, Ltd., Osaka Japan), and IL-7 (10 ng/mL; R&D Systems, Minneapolis, MN, USA). Half of the medium was replaced every 3–4 days with fresh AIM-V containing IL-2 and IL-7. On day 21, the cells were subjected to tetramer-based frequency analysis.

Tetramer-based CTL analysis. The proportion of peptide-specific CTLs was determined by tetramer staining. The HLA-A24/peptide tetramers were constructed by Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan). Cells were collected from each microwell and centrifuged then incubated with 50 nM of dasatinib (LC Laboratories, Woburn, MA, USA) for 30 min at 37°C. Subsequently, each tetramer was added and incubated for 30 min at room temperature. Then FITC-conjugated anti-CD8 antibody (Clone T8; Beckman Coulter, Brea, CA, USA) was added and incubated for another 20 min. The cells were washed in PBS and analyzed by flow cytometry using a FACS Caliber (Becton Dickinson, San Jose, CA, USA) and CellQuest software (Becton Dickinson). Living cells were gated and the proportions of tetramer-positive cells were calculated as the number of tetramer-positive cells/number of $CD8^+$ cells.

Statistical analysis. GraphPad Prism software version 7 was used for statistical analysis. Student's *t*-test was used to determine statistical differences. A value of $P < 0.05$ was considered statistically significant.

Results

The proportion of T_{YM} cells was decreased in sarcoma patients. At first, we investigated the proportions of $CD8^+$ T cell subsets consisting of T_N, T_{YM}, T_{SCM}, T_{CM} and T_{EM} cells in 23 healthy donors and 27 sarcoma patients (Tables 1 and 2). The proportion of T_N cells in sarcoma patients (mean proportion, 7.2%) was lower than that in healthy donors (24.2%; Fig. 1a). The proportion of T_{YM} cells in sarcoma patients (mean proportion, 9.0%) was also lower than that in healthy donors (17.08%; $P < 0.01$). The proportion of T_{YM} cells in sarcoma patients excluding young and elderly patients was also similarly low (Fig. S2). However, there were no significant differences between sarcoma patients and healthy donors in the proportions of T_{SCM} cells ($P = 0.67$), T_{CM} cells ($P = 0.31$) and T_{EM}

Table 1. Proportions of T cell subsets in 23 healthy donors

Heathy donors	Age	Gender	HLA-A24	% of CD8+ T cell				
				TN	TYM	TSCM	TCM	TEM
1	42	M	(+)	6.16	5.34	1.27	12.30	11.19
2	35	F	(+)	25.06	27.64	0.66	10.19	6.64
3	34	M	(+)	4.24	4.95	0.51	4.98	15.65
4	35	M	(+)	14.75	26.50	5.93	5.30	20.36
5	30	M	(+)	19.38	20.58	2.52	4.18	16.80
6	20	M	(−)	10.50	19.91	1.62	10.91	38.55
7	22	M	(−)	1.49	4.69	0.87	1.49	32.01
8	25	M	(+)	33.82	18.43	0.53	1.51	5.32
9	26	F	(−)	41.81	27.80	1.44	1.68	3.80
10	28	M	(+)	18.83	17.77	3.91	5.08	24.52
11	29	M	(+)	8.37	11.16	1.07	5.94	26.29
12	30	M	(+)	14.31	7.42	0.82	6.24	29.03
13	33	F	(−)	7.85	8.15	2.79	12.66	30.76
14	34	M	(−)	12.78	11.71	0.81	14.46	30.02
15	34	M	(−)	24.38	17.84	1.61	4.19	3.18
16	35	M	(+)	26.49	19.46	1.35	5.77	24.39
17	35	M	(+)	38.12	19.61	1.45	3.72	15.76
18	35	M	(+)	30.02	20.02	2.19	4.92	20.06
19	37	M	(−)	22.58	17.08	1.69	5.59	26.29
20	40	F	(−)	21.57	35.81	0.82	8.19	34.49
21	40	F	(+)	10.41	11.13	1.62	11.90	11.90
22	40	M	(+)	14.69	22.75	3.87	1.75	4.87
23	46	M	(+)	13.06	16.98	1.39	4.02	19.39

TCM, central memory T cells; TEM, effector memory T cells; TN, naïve T cells; TSCM, stem cell memory T cells; TYM, young memory T cells.

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Table 2. Proportions of T cell subsets in 27 patients with sarcoma

Patient	Age	Gender	Pathological diagnosis	Location	TMN Grade	HLA-A24	Chemotherapy	Prognosis	% of CD8+ T cell				
									TN	TYM	TSCM	TCM	TEM
1	40	F	Chondrosarcoma	Sacrum	3	(−)	Not done	DOD	1.99	3.91	1.2	1.48	16.28
2	55	F	Chondrosarcoma	Neck	2	(−)	Not done	CDF	4.93	4.75	0.48	3.26	25.33
3	74	F	Chondrosarcoma	Humerus	2	(+)	Not done	CDF	10.73	8.93	5.18	13.34	20.14
4	89	F	Chondrosarcoma	Femur	2	(+)	Not done	AWD	0.14	0.22	0.2	2.45	51.19
5	54	M	MPNST	Elbow	1	(−)	Not done	AWD	3.39	7.15	2.85	2.72	11.6
6	79	F	MPNST	Shoulder	1	(−)	Not done	DOD	7.5	10.44	1.55	6.37	14.65
7	57	F	Leiomyosarcoma	Lower leg	2	(−)	Not done	CDF	0.55	0.59	0.51	4.15	49.56
8	81	F	Myxofibrosarcoma	Forearm	1	(+)	Not done	CDF	3.7	2.65	0.52	12.12	23.15
9	4	F	Osteosarcoma	Femur	3	(−)	Not done	CDF	27.38	44.93	3.17	2.61	5.05
10	11	F	Osteosarcoma	Femur	3	(+)	Not done	AWD	15.52	12.87	0.89	4.46	20.53
11	15	M	Osteosarcoma	Femur	3	(+)	Not done	CDF	13.13	18.68	2.16	1.36	13.69
12	18	F	Osteosarcoma	Iliac bone	3	(−)	Not done	AWD	16.42	11.33	3	1.63	9.01
13	40	M	Osteosarcoma	Femur	3	(−)	Not done	AWD	7.47	3.68	4.53	6.17	21.97
14	24	F	Parosteal OS	Humerus	1	(+)	Not done	CDF	9.9	14.88	2.59	3.2	23.9
15	37	F	Parosteal OS	Humerus	1	(−)	Not done	CDF	13.9	14.32	4.42	3.4	21.87
16	19	M	Periosteal OS	Femur	2	(−)	Not done	Unknown	1.05	2.25	0.21	3.18	60.97
17	57	M	Synovial Sarcoma	Shoulder	3	(+)	Not done	Unknown	0.73	1.96	1.89	3.47	55.22
18	14	M	Epithelioid sarcoma	Lower leg	3	(−)	Done	CDF	8.88	16.86	2.43	2.23	17.14
19	23	F	Ewing Sarcoma	Femur	3	(+)	Done	CDF	7.57	8.83	7.09	5.45	11.63
20	83	M	Leiomyosarcoma	Thigh	2	(+)	Done	CDF	0.33	1.16	1.28	4.41	12.83
21†	57	F	Leiomyosarcoma	Lower leg	2	(−)	Done	CDF	0.36	0.32	1.11	8.94	53.76
22	55	M	MPNST	Scapular bone	2	(+)	Done	CDF	1.33	1.14	1.44	5.92	30.91
23	79	M	Myxofibrosarcoma	Thigh	1	(+)	Done	CDF	0.47	1.37	0.25	7.45	29.27
24‡	4	F	Osteosarcoma	Femur	3	(−)	Done	CDF	26.18	38.78	1.3	2.1	6.88
25	72	F	Undifferentiated pleomorphic sarcoma	Thigh	3	(−)	Done	CDF	6	6.02	1.78	17.79	26.15
26	66	M	Undifferentiated pleomorphic sarcoma	Forearm	3	(+)	Done	AWD	4.67	4.23	0.29	9.29	25.83
27	70	F	Undifferentiated pleomorphic sarcoma	Lower leg	2	(−)	Done	CDF	0.2	0.56	0.37	2.91	63.52

AWD, alive with disease; CDF, continuous disease free; DOD, died of disease; TCM, central memory T cells; TEM, effector memory T cells; TN, naïve T cells; TSCM, stem cell memory T cells; TYM, young memory T cells. †Patient 21 was identical to Patient 7 after chemotherapy. ‡Patient 24 was identical to Patient 9 after chemotherapy.

cells ($P = 0.09$). To investigate the effect of chemotherapy, we compared the proportions of T cell subsets in sarcoma patients who underwent chemotherapy and those who did not undergo. There was no significant difference between the proportion of T_{YM} or T_{SCM} cells, which have drug-resistance capacity, and the proportions of other T cell subsets (Fig. 1b).

Interestingly, only the memory proportion of T_{YM} cells was decreased in sarcoma patients but not in healthy donors. In addition, the proportions of T_{YM} cells were similar in sarcoma patients who received chemotherapy and those who did not. These findings suggest that the initiation of sarcoma might be due to the weakness of immune surveillance by T_{YM} cells and that chemotherapy does not impact the proportions of peripheral T cell subsets including T_{YM} cells.

T_{YM} cells of healthy donors memorized virus-derived antigens but not tumor-associated antigen. To investigate whether T_{YM} cells memorized viral antigens as did other T cell subsets in healthy donors, we performed mixed lymphocyte peptide culture (MLPC) using each of the T cell subsets of HLA-A24$^+$ healthy donors (Table 3). After MLPC, anti-Epstein Barr virus (EBV) CTLs were successfully induced from T_{YM} cells in 3 of 5 healthy donors, from T_{CM} cells in all five healthy donors and from T_{EM} cells in 3 of 5 healthy donors (Fig. 2a,b). In contrast, tumor-associated antigen-specific CTLs could not be induced from any of the T cell subsets.

T_{YM} cells of sarcoma patients who did not receive chemotherapy memorized both virus-derived antigens and tumor-associated antigens. Next, we performed MLPC using each of the T cell subsets of HLA-A24+ sarcoma patients who did not receive chemotherapy. Anti-EBV-specific CTLs were also induced from T_{YM} cells in 4 of 7 sarcoma patients, from T_{CM} cells in 3 of 7 sarcoma patients and from T_{EM} cells in 5 of 7 sarcoma patients who did not receive chemotherapy (Table 3, Fig. 2c, d). In contrast to healthy donors, CTLs directed to PBF were induced from T_{YM} cells in 3 of 7 sarcoma patients, from T_{CM} cells in 4 of 7 sarcoma patients and from T_{EM} cells in 3 of 7 of sarcoma patients who did not receive chemotherapy. Anti-survivin-2B-specific CTLs were also induced from T_{CM} cells

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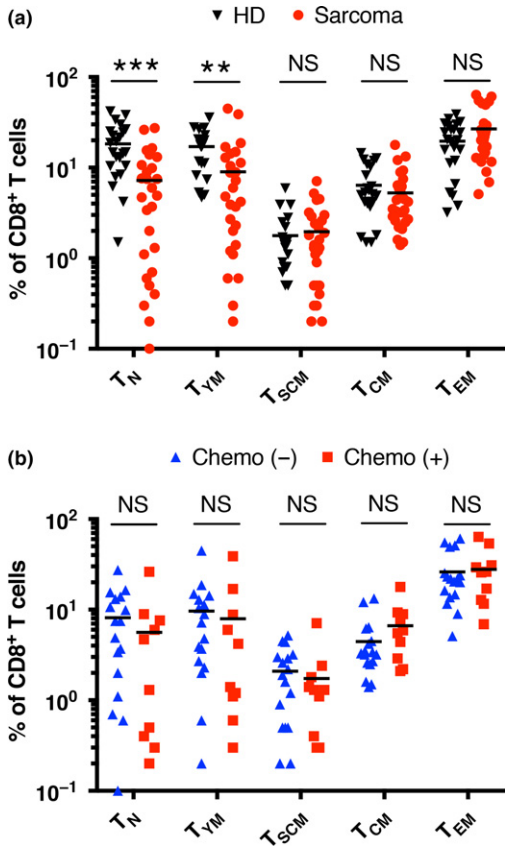

Fig. 1. Proportions of $CD8^+$ T cell subsets consisting of T_N, T_{YM}, T_{SCM}, T_{CM} and T_{EM} cells. (a) Proportions of T cell subsets in healthy donors and sarcoma patients. ***$P < 0.001$, **$P < 0.01$, NS; No significant difference. (b) Proportions of T cell subsets in sarcoma patient who received chemotherapy and those who did not.

in 1 of 7 sarcoma patients. These results suggested that T_{YM} cells might play an important role as well as T_{CM} and T_{EM} cells in the maintenance of memory function not only in healthy individuals but also in sarcoma patients.

Viral and tumor-associated antigen-specific T_{YM} cells were firstly recovered after chemotherapy in sarcoma patients. Finally, to investigate the effect of chemotherapy on memory T cell subsets, we performed MLPC using each of the T cell subsets of HLA-A24+ sarcoma patients who received chemotherapy. Despite chemotherapy, EBV-specific CTLs were induced from the memory T cell subsets including T_{YM} cells in 2 of 5 sarcoma patients and T_{CM} cells in 1 of 5 of sarcoma patients who received chemotherapy but were not induced from T_{EM} cells (Table 3, Fig. 2e). In Patient 22, anti-EBV-specific CTLs were induced from T_{YM} cells but not from T_{CM} or T_{EM} cells (Fig. 2f). These results suggested the importance of T_{YM} cells in recovery from the nadir and differentiation of memory subsets after chemotherapy. Regarding tumor-associated antigens, CTLs directed to PBF were induced from T_{YM} cells in 2 of 5 sarcoma patients but not from T_{CM} or T_{EM} cells in sarcoma patients who received chemotherapy. CTLs directed to survivin were induced from T_{YM} cells in 2 of 5 sarcoma patients from T_{CM} cells in 1 of 5 sarcoma patients but not and from T_{EM} cells in sarcoma patients who received chemotherapy (Table 3, Fig. 2f). Interestingly, in Patients 20 and 22, anti-PBF-specific CTLs were induced from T_{YM} cells but not from T_{CM} or T_{EM} cells (Fig. 2f). In Patient 22, anti-survivin-specific CTLs were induced from T_{YM} cells but not from T_{CM} or T_{EM} cells. These results support the idea that T_{YM} cells are resistant to chemotherapy and are firstly recovered to reconstitute the T cell memory subsets after the nadir condition. In contrast to T_{YM} cells, the function of T_{EM} cells was easily impaired by chemotherapy.

Table 3. Proportions of tetramer-positive cells after CTL induction in CD8+ T cell subsets of HLA-A24(+) healthy donors and sarcoma patients

	EBV			PBF			Survivin			HIV		
	TYM	TCM	TEM	TYM	TCM	TEM	TYM	TCM	TEM	TYM	TCM	TEM
Healthy donors (n = 5)												
HD 1	**0.21**	**2.78**	0.14	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
HD 2	**0.79**	**15.66**	**25.34**	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00
HD 3	0.00	**0.26**	0.00	0.00	0.02	0.05	0.00	0.05	0.00	0.00	0.00	0.00
HD 4	0.00	**5.15**	**3.83**	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
HD 5	**0.26**	**2.37**	**0.46**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sarcoma patients who did not receive chemotherapy (n = 7)												
Patient 3	**1.99**	**26.57**	**10.39**	0.14	**0.48**	**0.40**	0.00	0.06	0.02	0.00	0.00	0.00
Patient 4	0.02	0.13	**1.86**	0.07	0.03	**0.46**	0.17	0.11	0.09	0.00	0.00	0.00
Patient 8	**13.21**	**15.87**	**9.52**	0.16	**0.58**	0.20	0.12	**0.22**	0.05	0.00	0.00	0.00
Patient 10	0.00	0.00	0.00	**0.20**	**0.28**	0.00	0.10	0.02	0.02	0.00	0.00	0.00
Patient 11	**0.21**	0.17	0.02	**0.38**	**0.26**	**0.23**	0.00	0.06	0.04	0.00	0.00	0.00
Patient 14	**3.16**	**5.27**	**4.50**	**0.20**	0.09	0.02	0.05	0.11	0.02	0.00	0.00	0.00
Patient 17	0.00	0.18	**0.71**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sarcoma patients who received chemotherapy (n = 5)												
Patient 19	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.00
Patient 20	0.15	0.06	0.00	**0.32**	0.12	0.00	**0.34**	**0.22**	0.00	0.00	0.00	0.00
Patient 22	**0.38**	0.00	0.04	**0.65**	0.17	0.05	**0.36**	0.17	0.12	0.00	0.00	0.01
Patient 23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Patient 26	**0.21**	**1.34**	0.00	0.04	0.04	0.04	0.02	0.00	0.00	0.00	0.00	0.00

TCM, central memory T cells; TEM, effector memory T cells; TYM, young memory T cells. % tetramer-positive cells among CD8+ cells after mixed lymphocyte peptide culture with indicated peptide are shown. More than 0.20% of tetramer-positives were indicated in bold and underline.

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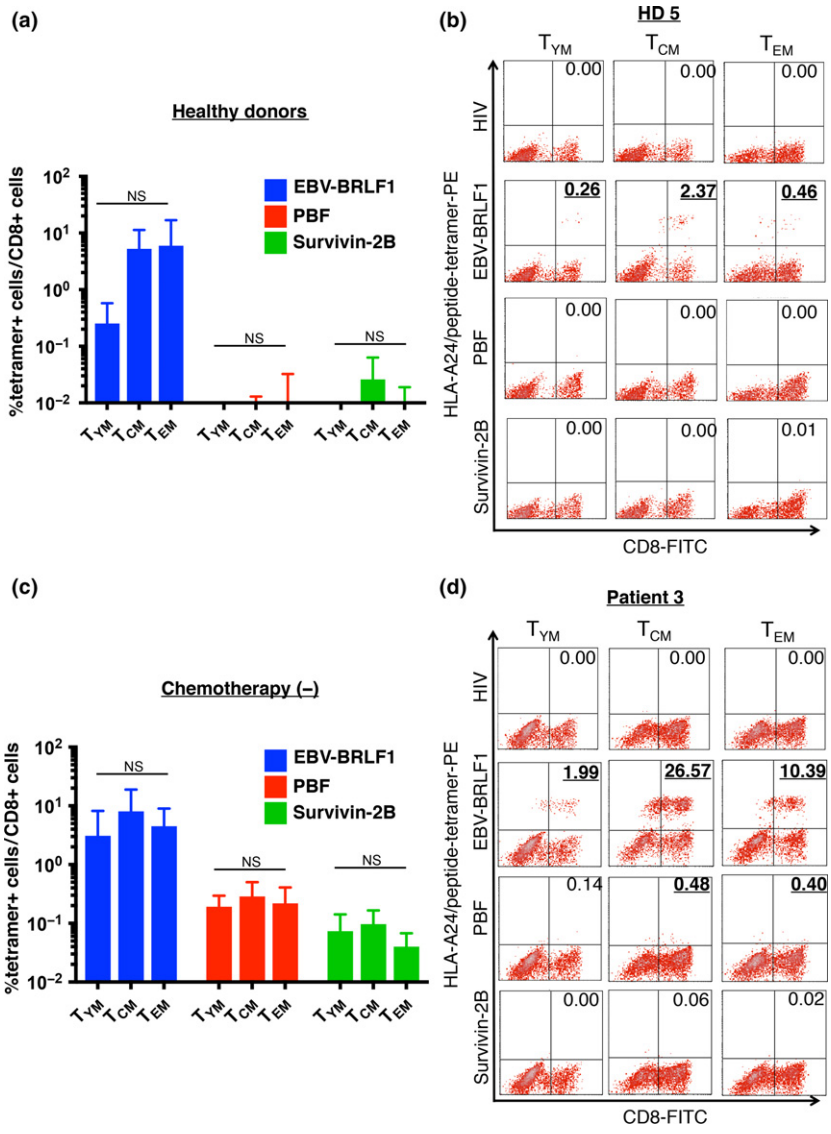

Fig. 2. Antigen-specific CTLs induced from peripheral T cell subsets from healthy donors and sarcoma patients who received and those who did not receive chemotherapy in the context of HLA-A24. Tetramer analysis after MLPC with the indicated peptides using sorted peripheral memory T cell subsets from healthy donors (a), sarcoma patients who did not receive chemotherapy (c) and sarcoma patients who received chemotherapy (e). *$P < 0.05$, NS: no significant difference. Results of tetramer analysis of a healthy donor (b: HD5), a sarcoma patient who did not receive chemotherapy (d: Patient 3), and sarcoma patients who received chemotherapy (f: Patients 20 and 22) are shown. More than 0.20% of tetramer-positives among CD8-positives are indicated in bold and underline.

Discussion

In this study, we demonstrated that: (i) T_{YM} cells existed in PBMCs of both healthy donors and sarcoma patients; (ii) the proportion of T_{YM} cells in sarcoma patients was lower than that in healthy donors; (iii) there was no difference in the proportion of any of the memory T cell subsets between patients who received chemotherapy and those who did not receive chemotherapy; (iv) viral antigen-specific CTL were induced from T_{YM} cells not only in healthy donors but also in sarcoma patients; and (v) tumor-associated antigens PBF and survivin-specific CTLs were induced from T_{YM} cells of sarcoma patients. It is notable that tumor-associated antigen-specific CTLs were weakly induced from T_{CM} cells and not from T_{EM} cells in sarcoma patients who received chemotherapy. These results suggested that T_{YM} cells were important for the maintenance of antigen-specific memory function to protect against pathogens and cancer cells not only in healthy donors but also in sarcoma patients, especially after chemotherapy.

The importance of memory T stem cells in self-renewal, long-lasting memory, proliferation and drug resistance has been widely recognized. T_{SCM} cells are useful for generating long-living T cells expressing TCR directed to tumor-associated antigens for adoptive cell transfer therapy.[9,10] The usefulness of peptide vaccination therapy targeting T_{SCM} cells has not been reported, and we consider that a vaccination strategy to enhance the immunological memory of T_{SCM} cells might be important.

In the present study, we focused on T_{YM} cells as a subset of memory T stem cells. T_{YM} cells express aldehyde dehydrogenase 1 (ALDH1) for drug resistance and are defined by naive markers ($CD73^+CD45RA^+CD62L^+CCR7^+$) and one memory marker ($CXCR3^+$) but lacking CD95. Expression status of surface markers suggested that T_{YM} cells might be located closer than T_{SCM} cells to naive cells in the T cell lineage and might be more important for the maintenance and reconstitution of T cell memory. A functional comparison of T_{YM} cells and T_{SCM} cells is essential. However, the proportion of T_{SCM} cells is much lower than T_{YM} cells in peripheral blood and they are difficult to isolate for functional analysis.

We observed that the peripheral proportion of T_{YM} cells in sarcoma patients was lower than that in healthy donors. In contrast to T_{YM} cells, the proportions of T_{SCM} cells were

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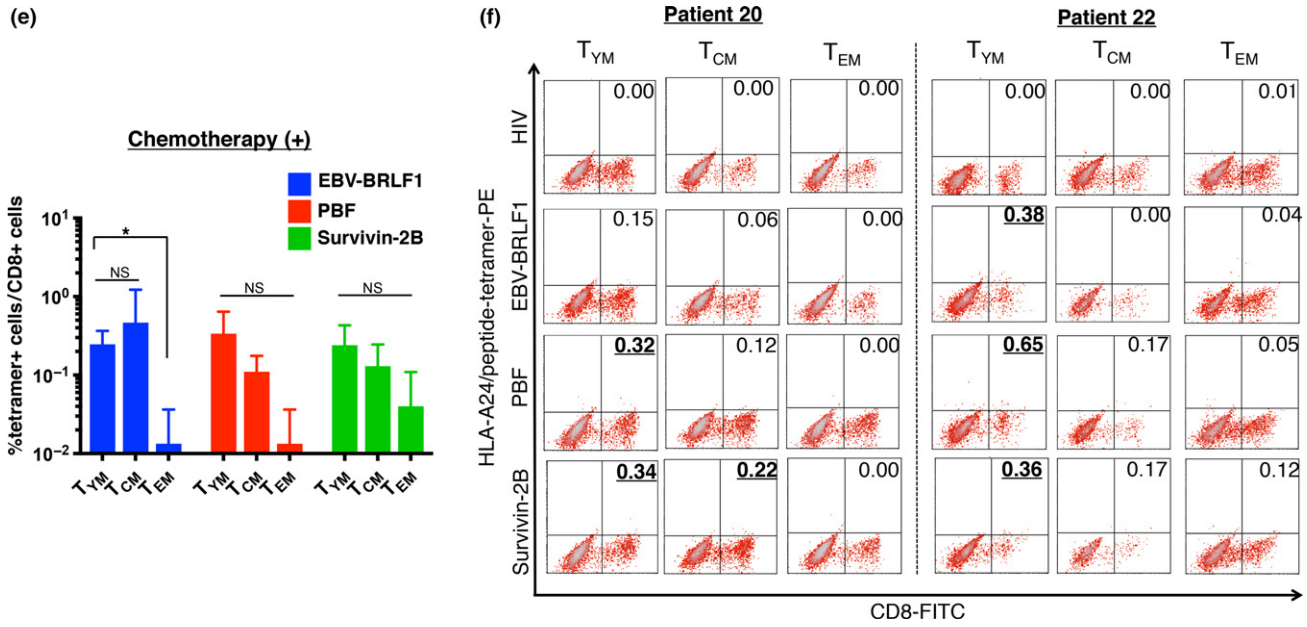

Fig. 2. Continued.

similar in sarcoma patients and healthy donors. These results suggest that the anti-tumor immunity conferred by T_{YM} cells is more important for preventing sarcoma initiation. Hong *et al.*[11] reported similar results showing that the peripheral proportion of $CD8^+$ T_{SCM} cells in lung cancer patients did not differ from that in healthy donors. We also assessed the relationship between proportion of T_{YM} cells and prognosis of the patients, but there was no difference in the proportion of T_{YM} cells or in the proportions of other T cell subsets in patients who were continuously disease free and those who were alive with disease or died of disease (Fig. S3). Generally, anti-tumor immune surveillance mainly plays a role in the tumor-initiation phase but is weakened after the establishment of tumor burden.[12] Therefore, the prognosis was not affected by the proportions of T cell subsets including T_{YM} cells.

Mixed lymphocyte peptide culture stimulated with the EBV peptide induced specific CTLs from not only T_{YM} cells but also T_{CM} and T_{EM} cells in healthy donors and sarcoma patients who did not receive chemotherapy. CTLs were induced with higher efficiency from T_{CM} cells and T_{EM} cells, than from T_{YM} cells. The capacity of T_{CM} cells and T_{EM} cells to differentiate to T_{EFF} cells is greater than that of T_{YM} cells because T_{CM} cells and T_{EM} cells are located more distal from T_{YM} cells in the T cell lineage.

Similar to healthy donors and sarcoma patients who did not receive chemotherapy, EBV-specific CTLs were induced from the T_{YM} fraction in sarcoma patients who received chemotherapy. These results suggested that T_{YM} cells have drug resistance capacity. PBMCs were collected at 3–4 weeks after preoperative chemotherapy. This period is required to finish the recovery from the nadir. The other T cell subsets might subsequently be recovered by differentiation from T_{YM} cells.

Tumor-associated antigen-specific CTLs could be induced from the T_{YM} fraction in sarcoma patients who received chemotherapy. These results indicate a possible beneficial effect of the use of T_{YM} cells for peptide vaccination in patients who receive chemotherapy. However, the proportions of induced CTLs directed to tumor-associated antigens by MLPC were still low, <1%. To overcome this problem, the development of efficient CTL induction from T_{YM} cells or an *ex vivo* expansion technique of T_{YM} cells is required. Cieri *et al.* generated T_{SCM} from naive subsets using CD3 and CD28 stimulation and culture with IL-7 and IL-15 cytokines.[13] However, the resultant T_{SCM} cells expressed both CD45RA and CD45RO. Expanded T_{SCM} cells might differ from original T_{SCM} cells. In a previous study, we stimulated T_N and T_{YM} fractions lacking CD95 with CD3/CD28 microbeads and cultured them with IL-7 and IL-15. As a result, T_N and T_{YM} fractions expressed CD95.[6] Therefore, *ex vivo* expansion of T_{YM} cells without change in the original character might be difficult. The proportion of T_{YM} cells was higher than that of T_{SCM} cells. T_{YM} cells might enable the production of large amounts of long-living tumor-specific CTLs for adoptive transfer therapy.

Vaccination with novel tumor-associated antigens is one possible option for enhancing anti-tumor immunological memory. Chemo-resistant tumor cells with strong tumor-initiating ability play an important role in recurrence and metastasis after chemotherapy.[14] Such therapy-resistant tumor cells are called cancer stem-like cells/cancer-initiating cells (CSCs/CICs). We previously reported that tumor-associated antigens (DNAJB8, or7c1 and ASB4) were highly expressed in CSCs/CICs. Immunotherapy targeting CSCs/CISs might also be attractive for sarcoma patients who received chemotherapy.[15] Therefore, we performed MLPC with CTL epitopes of DNAJB8 (AFMEAFSSF),[16] or7c1 (TYAGCLSQIF)[17] and ASB4 (IYPPQFHKV; S. Miyamoto, V. Kochin, T. Kanaseki, A. Hongo, S. Tokita, Y. Kikuchi, A. Takaya, Y. Hirohashi, T. Tsukahara, T. Terui, K. Ishitani, F. Hata, I. Takemasa, A. Miyazaki, H. Hiratsuka, N. Sato and T. Torigoe, unpublished data) in the context of HLA-A24 using four healthy donors and three sarcoma patients who did not receive chemotherapy (HD 1, HD 2, HD 3, HD 4, Patient 8 Patient 11 and Patient 14). From sarcoma patients who received chemotherapy, we could not obtain a sufficient number of PBMCs to perform MLPC. As a result, CSC/CIS antigen-specific CTLs were hardly observed after MLPC from both healthy donors and sarcoma patients who did not

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receive chemotherapy (data not shown). Since we successfully obtained CTL clones directed to each epitope in previous studies, immunogenicity of these antigens is promising. Clinical peptide vaccination trials are required to evaluate the immunogenicity directed to CSC/CIC antigens in T_{YM} cells and other T cell subsets.

In conclusion, T_{YM} cells existed and play an important role in anti-virus immune function in sarcoma patients and healthy donors. Moreover, T_{YM} cells have memory directed to tumor-associated antigens in patients with sarcoma who received chemotherapy and those who did not. Finally, the characteristics of T_{YM} cells might be useful for peptide vaccination and adaptive cell transfer in the future.

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Disclosure Statement

The authors declare no commercial or financial conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Gating strategy to define the CD8+ T cell subsets.

Fig. S2. Proportions of CD8+ T cell subsets in healthy donors and sarcoma patients (20–60 years).

Fig. S3. Proportions of CD8+ T cell subsets in the sarcoma patients who were continuously disease-free and others who were alive with disease or died of disease.

Table S1. Fluorescent antibodies.

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