Supporting Information

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SI Results

Treg Frequency Did Not Correlate with NY-ESO-1–Specific IFN-γ Production. To exclude the possibility that the impaired IFN-γ production by tetramer$^+$ cells is mediated by CD4$^+$CD25$^+$FOXP3$^+$ Tregs, we examined the CD8/Treg ratio in these specimens as previously described (Fig. S2*A*) (1). We found that Treg frequency and CD8/Treg ratio in tumor-infiltrating lymphocytes did not correlate with IFN-γ production by NY-ESO-1–specific CD8$^+$ T cells (Fig. S2*B*).

LAG-3$^+$CD8$^+$ T Cells Preferentially Accumulate at the Ovarian Tumor Site. Because of the striking negative association between LAG-3 expression and effector function of NY-ESO-1–specific T cells, we sought to determine the distribution of LAG-3$^+$CD8$^+$ T cells in the periphery and tumors of epithelial ovarian cancer (EOC) patients. We assessed bulk CD8$^+$ T cells for the frequencies of LAG-3$^+$CD8$^+$ T cells in peripheral blood lymphocytes (PBLs) from healthy donors, PBLs from ovarian cancer patients, tumor-associated lymphocytes (TALs), and tumor-infiltrating lymphocytes (TILs). The frequency of LAG-3$^+$CD8$^+$ T cells in normal donor PBLs was similar to ovarian cancer patients' PBLs. In contrast, TILs and TALs contained significantly more LAG-3$^+$CD8$^+$ T cells compared with PBLs from healthy individuals and EOC patients (Fig. S3 *A* and *B*). Because it has been reported that LAG-3 expression can also be detected by intracellular staining (2), we assessed cytoplasmic LAG-3 expression in these samples. Using this approach, we found that the expression level of intracellular LAG-3 in TILs and TALs was significantly higher than that for PBLs (Fig. S4 *A* and *B*). Together, these results indicate that LAG-3$^+$CD8$^+$ cells were significantly enriched at the ovarian tumor site.

Phenotype of LAG-3$^+$CD8$^+$ Cells at the Tumor Site Is CD127^{low} and CTLA-4^{high}. Regarding CD127, we found that in contrast to healthy donor and EOC PBLs, the majority of CD8$^+$ T cells in TILs and TALs were CD127^{low}, consistent with the exhausted functional phenotype. In addition, LAG-3$^+$ cells in TILs and TALs showed higher expression of CTLA-4 compared with whole CD8$^+$ T cells. However, there were no significant differences in the expression of CD28, CD62L, and CD45RO between LAG-3$^+$ and whole CD8$^+$ T cells (Fig. S5 *A* and *B*).

APCs Derived from TILs Up-Regulated PD-1 Expression and Inhibited Proliferation of an NY-ESO-1–Specific CD8 Clone. Because it has previously been shown that almost all ovarian tumors express PD-L1 and PD-L2 (3), the possibility existed that engagement of PD-1 on tumor-infiltrating CD8$^+$ T cells in vivo by tumor cells, and LAG-3 by MHC class II, a candidate ligand for LAG-3, on tumors or tumor-derived antigen-presenting cells (APCs) could lead to T-cell hyporesponsiveness. To test this notion, we stimulated an HLA-Cw*03-restricted NY-ESO-1$_{92–100}$-specific CD8 clone, RPOV10, derived from TILs of a seropositive EOC patient, with peptide-pulsed autologous PBL-derived APCs, tumor-derived APCs, or a combination of these cells. Similar to results obtained from polyclonal NY-ESO-1–specific T cells (4), we found that PD-1 expression was augmented on clone RPOV10 in the presence of tumor-derived APCs. Moreover, these cells stimulated by tumor-derived APCs exhibited slower proliferation as determined by carboxyfluorescein succinimidyl ester (CFSE) dilution, compared with RPOV10 stimulated with PBL-derived APCs (Fig. S7).

SI Materials and Methods

Ex Vivo Staining. Cells isolated from PBLs, TILs, or TALs were stained with NY-ESO-1 tetramers and mAbs against CD8, CTLA-4, CD45RO, CD62L, and CCR7 (BD Biosciences), and analyzed by FACSCalibur (BD Biosciences).

Immunohistochemistry. Immunohistochemistry of tumor tissue was performed as described previously (1). Briefly, tumor specimens were fixed with buffered formalin and embedded in paraffin. The sections of paraffin block were deparaffinized, pretreated with buffer, inactivated endogenous peroxidase, and blocked with serum-free protein block (DakoCytomation). Then the samples were stained with anti-CD25 (Vector Labs), anti-FoxP3 (Biolegend), anti-CD8 (DakoCytomation), and anti-CD4 (DakoCytomation) antibodies and developed with secondary-antibody-conjugated enzyme, followed by counterstaining with hematoxylin solution. Negative control slides omitting the primary antibody were included in all assays. The number of positively stained cells was counted.

Cytokine Production from CD8$^+$ Cells. CD8$^+$ cells were isolated from PBLs, TALs, or TILs using a Dynal CD8 Positive Isolation Kit (Invitrogen). These purified CD8$^+$ cells were stimulated with beads coated with anti-CD3/anti-CD28 mAbs (Invitrogen) in complete medium. Twenty-four hours later, the culture supernatant was collected and cytokine levels were measured by ELISA (eBioscience).

Proliferation of CD8 Clone. An established T-cell line (2×10^5 cells) that was specific for NY-ESO-1$_{92–100}$ peptide was labeled with CFSE and stimulated with NY-ESO-1$_{92–100}$-pulsed autologous CD8$^-$CD4$^-$ cells (1×10^6 cells) derived from PBLs, TILs, or a 1:1 mixture. Seven days later, PD-1 expression and CFSE dilution were analyzed by gating on tetramer$^+$ cells.

Cytokine Treatment. PBLs from healthy donors were cultured with different concentrations of recombinant human IL-10, IL-6, TGF-β (eBioscience), or a combination of these cytokines for 4 days. PD-1 and LAG-3 expression on CD8$^+$ T cells was determined by flow cytometry.

1. Sato E, et al. (2005) Intraepithelial CD8$^+$ tumor-infiltrating lymphocytes and a high CD8$^+$/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci USA* 102:18538–18543.
2. Grosso JF, et al. (2007) LAG-3 regulates CD8$^+$ T cell accumulation and effector function in murine self- and tumor-tolerance systems. *J Clin Invest* 117:3383–3392.
3. Hamanishi J, et al. (2007) Programmed cell death 1 ligand 1 and tumor-infiltrating CD8$^+$ T lymphocytes are prognostic factors of human ovarian cancer. *Proc Natl Acad Sci USA* 104:3360–3365.
4. Huard B, Prigent P, Tournier M, Bruniquel D, Triebel F (1995) CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins. *Eur J Immunol* 25:2718–2721.

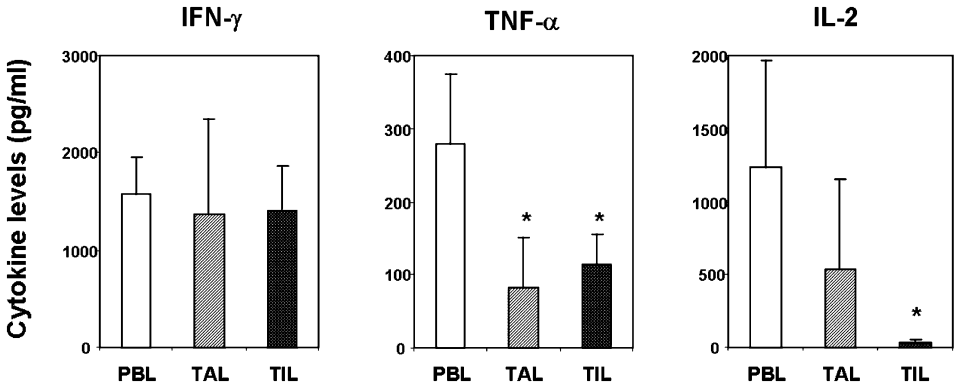


Fig. S1. Cytokine production from $CD8^+$ cells in PBLs, TALs, or TILs of five EOC patients. $CD8^+$ cells were isolated from specimens and stimulated with anti-CD3/anti-CD28 mAb-coated beads for 24 h. The cytokine levels in the supernatant were measured by ELISA. $^*P < 0.05$ compared with PBLs.

A

	CD8	CD4	$CD25^+FoxP3^+$	CD8/CD4 ratio	CD8/Treg ratio
#13076	25	24	14	1.0	1.8
#13566	45	8	2	5.6	22.5
#16520	76	35	23	2.2	3.3
#17383	28	31	20	0.9	1.4
#17505	48	45	28	1.1	1.7
#18098	17	6	7	2.8	2.4

B

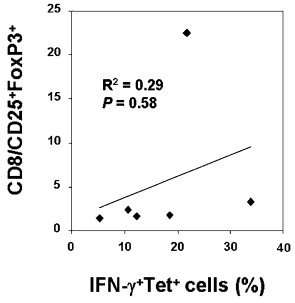


Fig. S2. The frequency of regulatory T cells was not involved in NY-ESO-1–specific IFN-γ production. (*A*) The number of $CD8^+$, $CD4^+$, and $CD25^+FoxP3^+$ cells at the tumor site was determined by immunohistochemistry. (*B*) Correlation between NY-ESO-1–specific IFN-γ production and $CD8^+/CD25^+FoxP3^+$ ratio. The coefficient of correlation (R^2) and *P* value are shown.

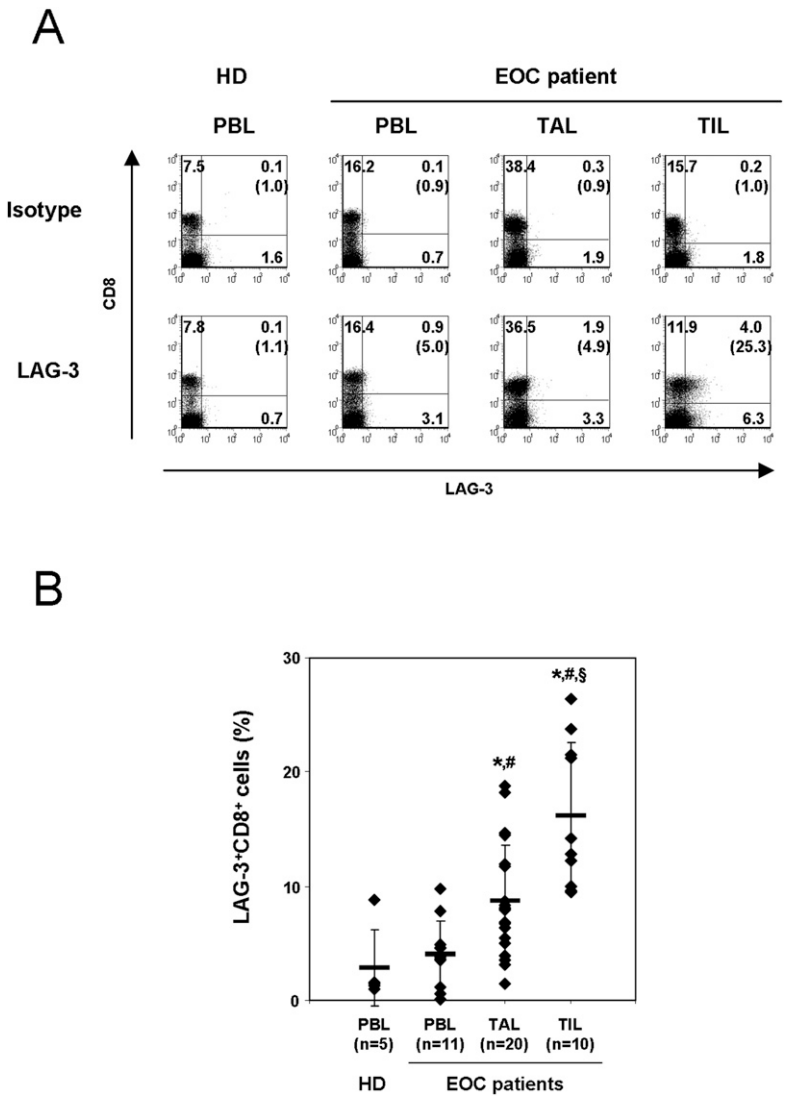
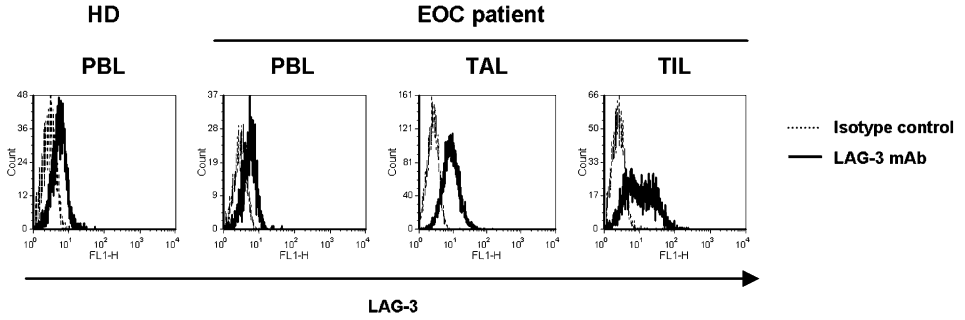


Fig. S3. LAG-3 expression is up-regulated at the tumor site of EOC patients. (*A*) PBLs, TALs, or TILs were stained with anti-LAG-3 or isotype-matched antibodies and analyzed by flow cytometry. Numbers in parentheses represent the percentage of LAG-3$^+$CD8$^+$ T cells. (*B*) Summary of the proportions of LAG-3$^+$CD8$^+$ cells in samples from several healthy donors (HD) and EOC patients. *$P < 0.05$ compared with healthy donors' PBL. $^{\#}P < 0.05$ compared with EOC patients' PBL. $^{\S}P < 0.05$ compared with TALs.

A



B

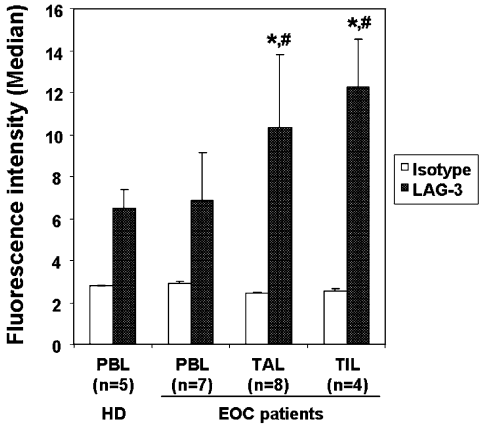


Fig. S4. Cytoplasmic LAG-3 expression-level increase in TALs and TILs. (*A*) Intracellular LAG-3 expression in $CD8^+$ T cells from healthy donors or EOC patients was analyzed ex vivo. (*B*) The median intensities of cytoplasmic LAG-3 staining compared for PBLs from healthy donors and PBLs, TILs, and TALs from EOC patients. $^*P < 0.05$ compared with healthy donors' PBLs. $^{\#}P < 0.05$ compared with EOC patients' PBLs.

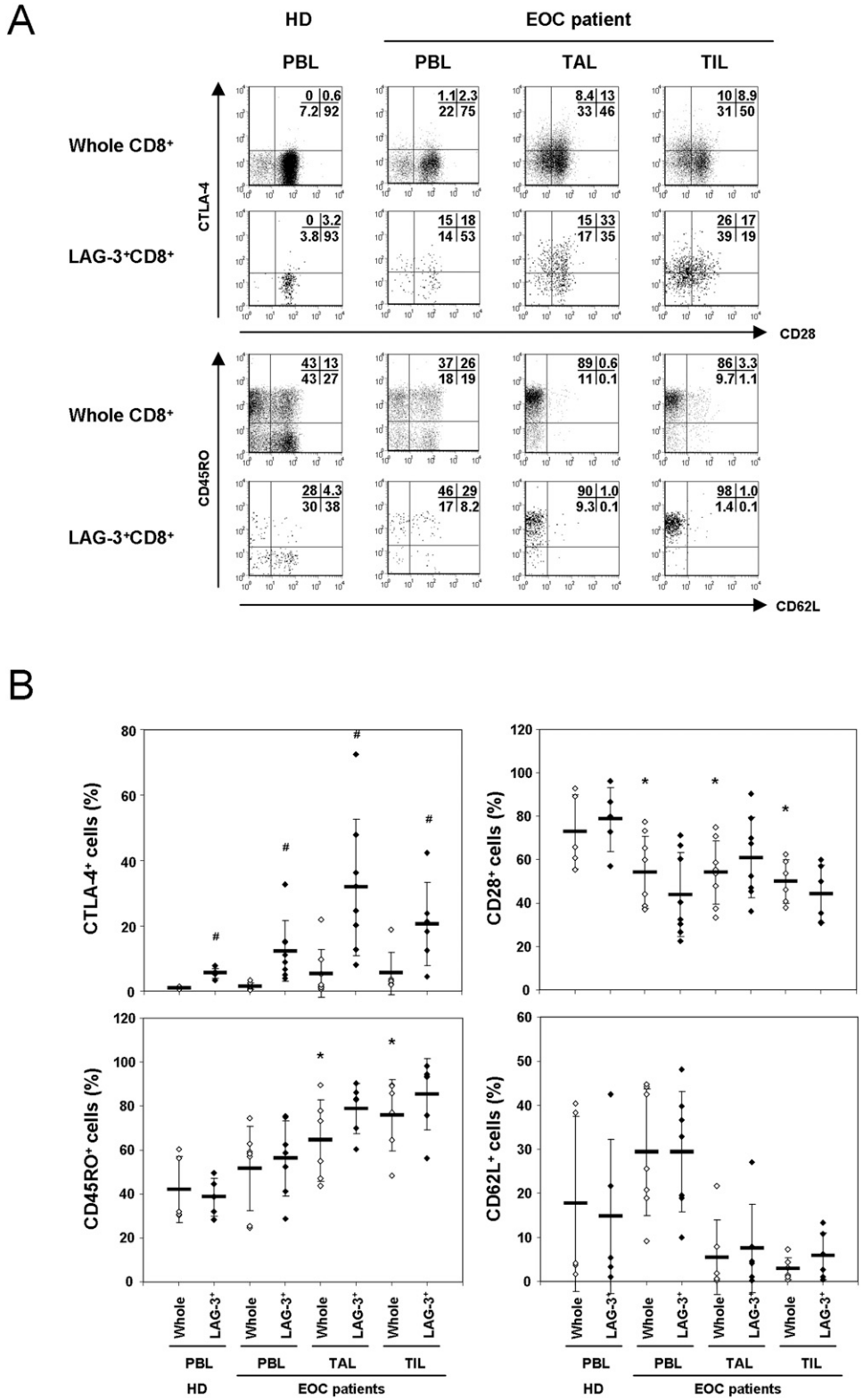
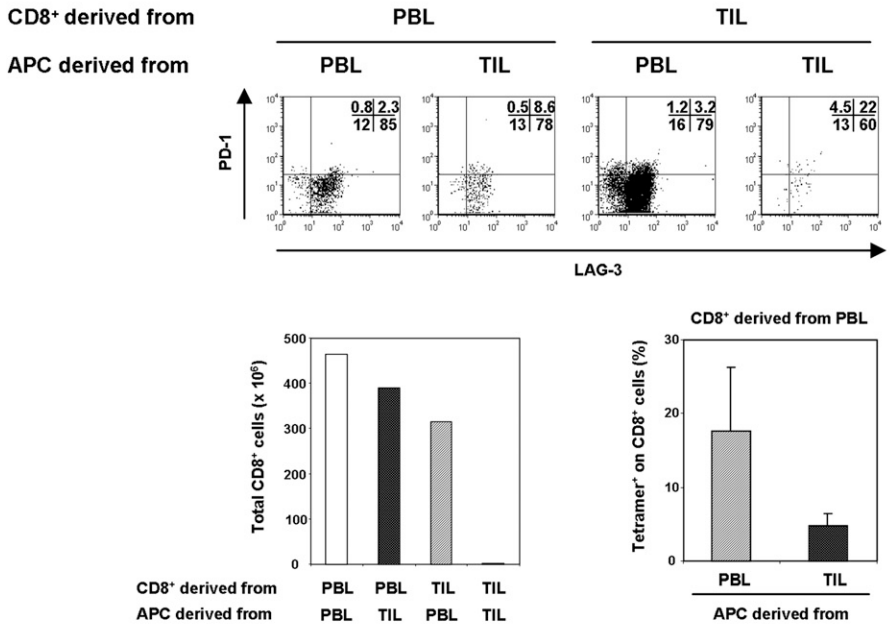


Fig. S5. The phenotypic characterization of LAG-3$^+$CD8$^+$ cells in PBLs, TALs, or TILs. (*A*) The surface expression of CTLA-4, CD28, CD45RO, and CD62L on whole CD8$^+$ or LAG-3$^+$CD8$^+$ cells. Numbers indicate the percentage of cells in each quadrant. (*B*) The expression of indicated molecules from all specimens. *$P < 0.05$ compared with whole CD8$^+$ cells of healthy donors' PBLs. $^{\#}P < 0.05$ compared with whole CD8$^+$ cells of each corresponding tissue.

A



B

The frequency of LAG-3$^+$PD-1$^+$CD8$^+$ cells after *in vitro* culture

		APC derived from PBL	APC derived from TIL
CD8 derived from	PBL	+	++
	TIL	+	++++

Fig. S6. LAG-3$^+$PD-1$^+$CD8$^+$ cells are increased by culture with APCs derived from TILs. (*A*) CD8$^+$ cells derived from PBLs or TILs were cultured with peptide-pulsed APCs derived from PBLs or TILs. Ten to 15 days later, the expression of LAG-3 and PD-1, the number of CD8$^+$ cells, and tetramer frequency were determined. (*B*) Summary of the frequency of LAG-3$^+$PD-1$^+$ cells after in vitro culture of CD8$^+$ cells with APCs derived from PBLs or TILs.

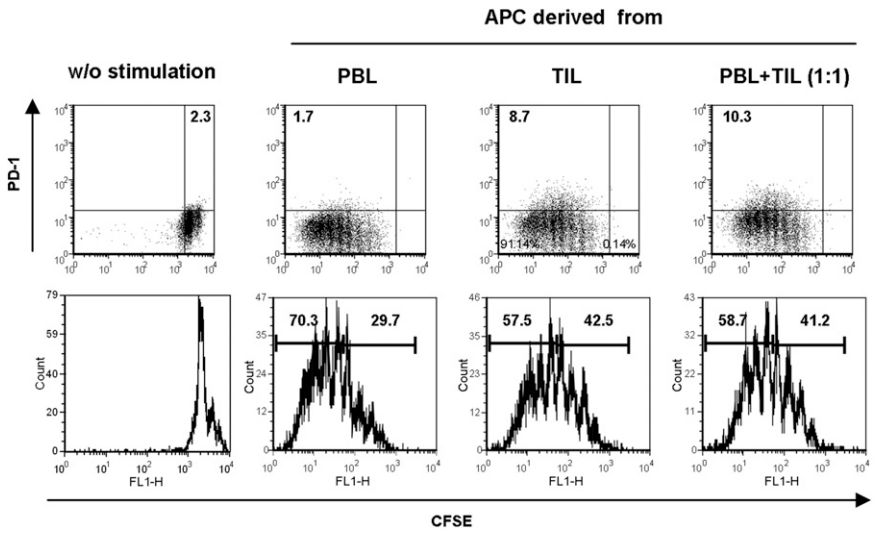
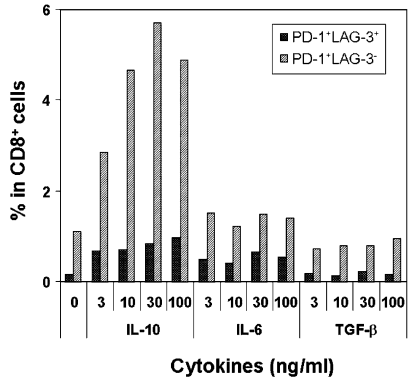


Fig. S7. Induction of PD-1 expression on $CD8^+$ T cells by antigen stimulation with tumor-associated APCs. An NY-ESO-$1_{92–100}$-specific $CD8^+$ clone was stimulated with peptide-pulsed autologous $CD8^-CD4^-$ cells derived from PBLs, TILs, or a 1:1 mixture. After 7 days of culture, CFSE dilution and PD-1 expression on $tetramer^+CD8^+$ T cells were determined. The result shown is representative of two independent experiments.

A

Cytokine	pg/ml	Range
IL-10	131.2 ± 83.4	38.3 – 267.9
IL-6	12110.3 ± 7204.4	2379.4 – 29344.6
TGF-β	655.5 ± 353.7	269.9 – 1502.2
IFN-γ	48.6 ± 53.5	8.0 – 176.3

B



C

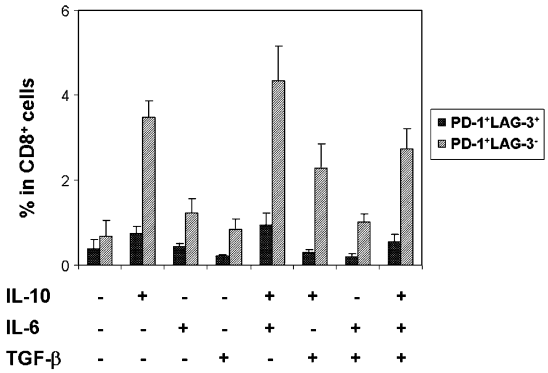


Fig. S8. IL-10 and IL-6 induce LAG-3$^+$PD-1$^+$ and LAG-3$^-$PD-1$^+$CD8$^+$ T cells. (*A*) The cytokine levels in ascites fluids from 16 patients were determined by ELISA. (*B*) PD-1 and LAG-3 expression on PBLs of healthy donors was examined after culture with graded amounts of IL-10, IL-6, or TGF-β for 4 days. (*C*) The effect of the combination of cytokines on PD-1 and LAG-3 expression. IL-10 (30 ng/mL), IL-6 (10 ng/mL), and TGF-β (10 ng/mL) were added to the culture. Results shown are representative of two independent experiments.

Table S1. Proportion of tetramer$^+$ cells in TILs or TALs derived from NY-ESO-1–seropositive patients

Patient number	Serum NY-ESO-1 Ab titer	HLA class I type						Specimen	NY-ESO-1–specific tet$^+$ cells (%)
		A1	A2	B1	B2	C1	C2		
13076	35163	***02**	*68	*39	*51	*05	*12	TIL	2.34
13566	35054	*03	*11	NT	NT	***03**	*05	TIL	16.4
								TAL	3.85
14035	14624	***02**	–	*44	*51	*0501	*1502	TIL	0.01
								TAL	0
14211	59953	*0301	*2601	*0702	*1501	***0303**	*0702	TIL	0
15936	102638	***0201**	–	*3801	*5001	*0602	*1203	TIL	12.5
								TAL	0.45
16253	27604	***0201**	*0301	*0401	–	***0304**	–	TAL	0
16300	381001	***0201**	*0301	*0702	*4402	*0501	*0702	TIL	0
								TAL	0
16520	15975	***0201**	*0302	*1501	*4101	***0303**	*1701	TIL	26.2 (Cw3)
17104	50913252	***0201**	*3201	*0702	*3501	*1401	*0702	TIL	0.46
								TAL	0.26
17383	20482	*0302	*1101	*0801	*4001	***0304**	*0701	TIL	20.9
17457	2518	***0201**	*2402	*0702	*4405	*0202	*0702	TIL	0
								TAL	0
17578	20482	*0101	***0201**	*0801	*1501	***0304**	*0701	TIL	0.99 (A2)
									0.64 (Cw3)
17505	1000000	*01	*3201	*1501	*35	*0202	***0303**	TIL	2.32
18098	1000000	*11	*29	*1801	*5501	***03**	–	TIL	5.75
								TAL	0.51

NT, not tested.

Table S2. The majority of NY-ESO-1-seropositive patients have detectable NY-ESO-1–specific CD8$^+$ cells in PBLs following in vitro stimulation

Patient number	NY-ESO-1-specific tet$^+$ cells (%)	IFN-γ^+ tet$^+$ cells	NY-ESO-1-specific CD8$^+$ cells (detected by overlapping peptides)
00012	0 (A2)	0	–
00017	NA	NA	–
12351	NA	NA	–
13076	2.14 (A2)	30.8	+
13339	NA	NA	–
13534	2.88	NT	+
13754	0.21 (A2)	NT	+
13987	NA	NA	+
14035	0 (A2)	0	+
14174	NA	NA	+
14983	NA	NA	–
15670	NA	NA	–
15775	NA	NA	–
15867	NA	NA	+
15936	0.41 (A2)	79.6	+
16253	0 (A2)	0	–
16284	NT (A2)	NT	+
16520	21.4 (Cw3)	52.6	+
17383	2.14 (Cw3)	54.9	+
17505	1.17 (Cw3)	62.6	+
17578	2.74 (Cw3)	84.3	+
18098	0.20 (Cw3)	NT	+
9280445	NA	NA	+

NA, tetramer not available (patients are not HLA-A2$^+$ or HLA-Cw3$^+$); NT, not tested.