SUPPLEMENTAL MATERIAL

Huang et al., http://www.jem.org/cgi/content/full/jem.20090397/DC1

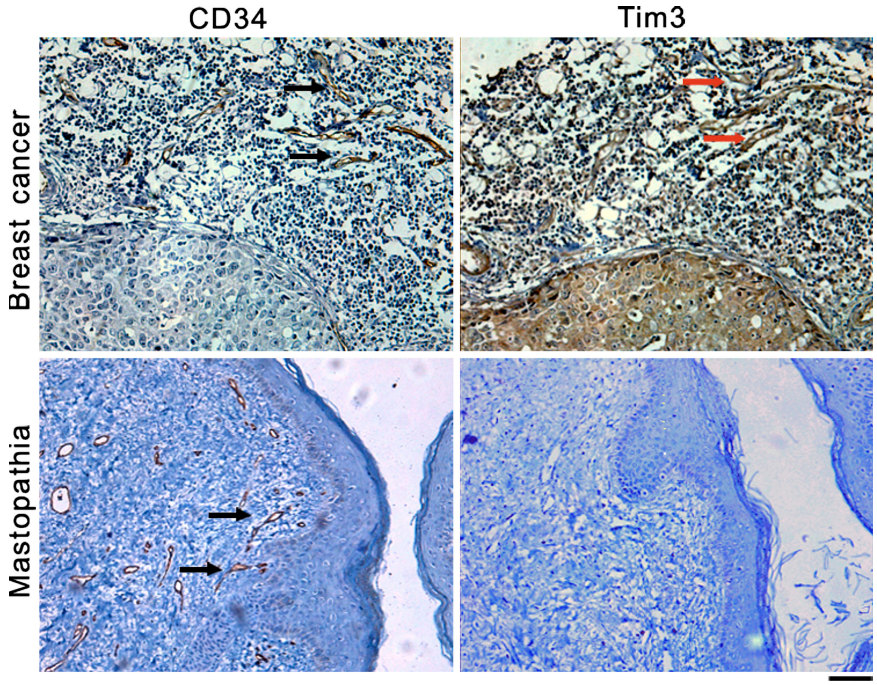

Figure S1. Tim-3 is preferentially expressed on endothelium in breast cancer tissues. Immunohistochemical staining of CD34 (left) and Tim-3 (right) proteins was performed in mastopathia (n = 40) or breast cancer sections (n = 20). The representative images depict similar data observed in most of the samples examined. Immunoreactive Tim-3 (red arrows) was readily detectable in endothelium (black arrows) of breast cancer but not of mastopathia tissues. Bar, 20 µm.

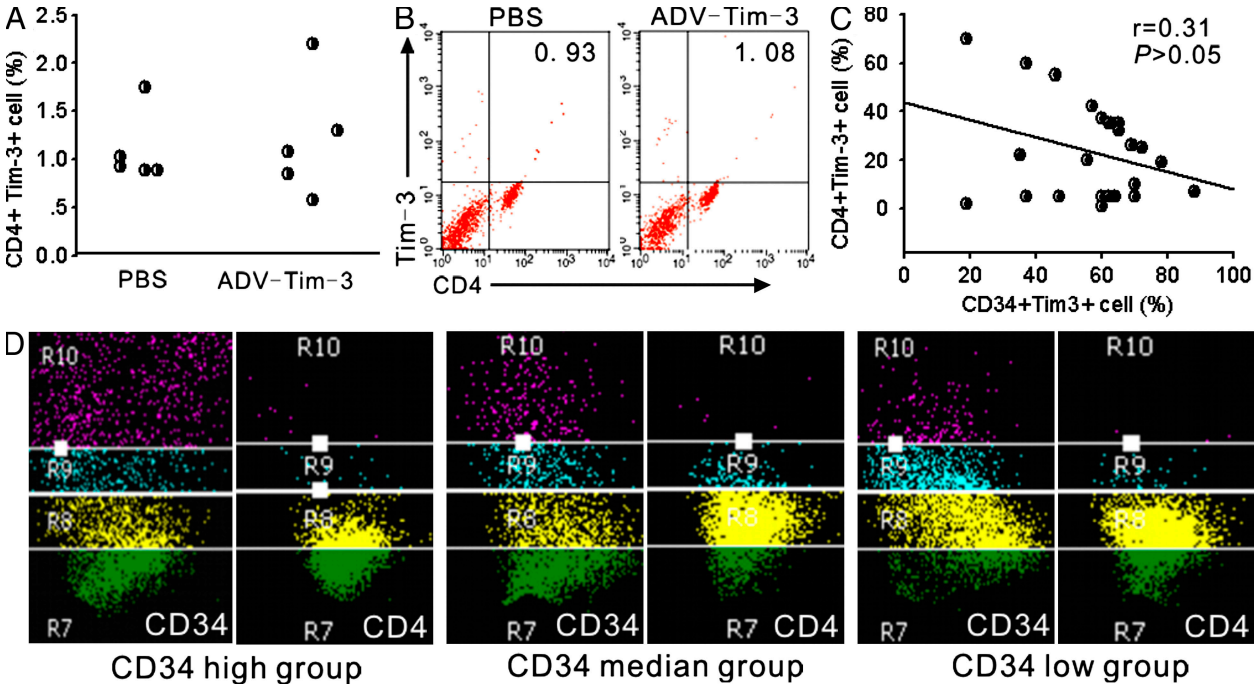

Figure S2. The possible correlation of CD34⁺Tim-3⁺ ECs with CD4⁺Tim-3⁺ T cells in lymphoma samples. (A) ADV–Tim-3–infected UVECs were incubated with autologous lymphocytes. Lymphocytes were then subjected to flow cytometry analysis for the percentages of CD4⁺Tim-3⁺ cells. Each data point represents the mean ± SE percentage of triplicates. (B) Representative images of flow cytometry analysis depict similar data in all other samples examined. Numbers represent the percentages of CD4⁺Tim-3⁺ cells. (C) To determine numbers of CD34⁺Tim-3⁺ ECs or CD4⁺Tim-3⁺ T cells in lymphoma tissues sections, four lymphoma samples were subjected to analysis by laser scanning cytometry (LSC; Compucytes). The Tim-3⁺ cells were initially labeled and gated under a low-power field. In the next continuous sections, numbers of CD34⁺ or CD4⁺ cells were counted within the gated Tim-3⁺ areas and calculated as proportions of the total Tim-3⁺ cell population. Each data point represents the percentage of CD4⁺Tim-3⁺ T cells and CD34⁺Tim-3⁺ ECs in a randomly selected area from the lymphoma tissue section. Six areas were randomly selected for analysis from each tissue section. Scatter plots show CD34⁺Tim-3⁺ ECs versus CD4⁺Tim-3⁺ T cells in 24 selected areas from four lymphoma tissues sections. Levels of Tim-3 protein in ECs did not correlate with Tim-3 expression of the CD4⁺ T cells in lymphoma tissue sections as defined by LSC ($r = 0.31$; $P > 0.05$). (D) Three typical results of laser scanning cytometry analysis depict similar data in all other samples examined. Cell populations including R9 + R10 represent gated total Tim-3⁺ cells. The gated cells were then counted for CD34⁺ or CD4⁺ cells by laser scanning cytometry analysis. R7, R8, R9, or R10 represents various immunoreactive intensities of CD4 or CD34 antigens, and cell populations within R9 or R10 regions were calculated as CD4 or CD34 positive (R7, negative; R8, weak immunoreactivity; R9, moderate immunoreactivity; R10, high immunoreactivity). Similar results were observed in three (C and D) independent experiments.

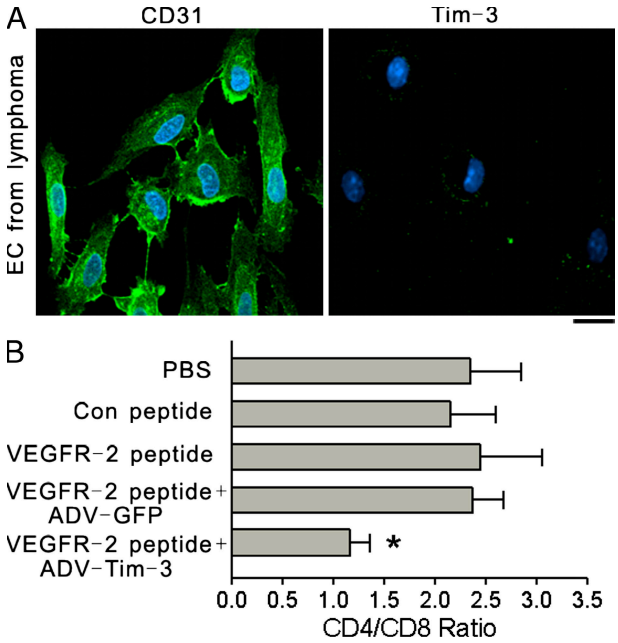

Figure S3. Tim-3–expressing ECs derived from primary lymphoma tissues suppress antigen-induced activation of CD4⁺ T cells. HLA-DR alleles of lymphoma patients were determined by high-resolution molecular HLA typing methods. Three newly diagnosed lymphomas with the HLA-DRB1*1501 (n = 2) or HLA-DRB1*0701 (n = 1) subtype were included in the experiments. ECs were purified from fresh lymphoma tissue and amplified in M199 supplemented with 20% FCS, 10 ng/ml of epithelial growth factor, 35 µg/ml gentamycin, 1 µg/ml hydrocortisone, and 2.5 µg/ml amphotericin B for >15 d. Next, ECs were digested using 0.25% trypsin and 1 mM EDTA, seeded onto uncoated 24-well plates, pulsed with a 15-mer peptide mapping to the 167–181 region of VEGFR-2 (EKRFVPDGNRISWDS), and infected with various adenoviral mutants at an MOI of 250 for 2 d. An unrelated sequence (VALWLCVPTRAASVG) was designed as a control peptide. Autologous PBMCs were obtained from heparinized peripheral blood of the same patients. To study the interaction of ECs with autologous T cells, autologous PBMCs were mixed with ECs at a ratio of 10:1 in the presence of 50 U/ml IL-2 for 10 d. The lymphocytes were then analyzed by FACS analysis for expression of CD3, CD4, and CD8. (A, left) After >15 d of culture, the purity of ECs was confirmed by positive staining for CD31 (green) and observed with a confocal laser scanning microscope. Cell nuclei (blue) were visualized by staining with DAPI. (right) After >15 d culture, ECs derived from fresh lymphoma tissues were detected with negative expression of Tim-3. Bar, 100 µm. (B) Expression of Tim-3 in ECs significantly decreased the CD4/CD8 ratio in cultured autologous PBMCs. *, $P < 0.01$ compared with all other groups. Data are represented as means ± SD of six experiments. Similar results were observed in three independent experiments.

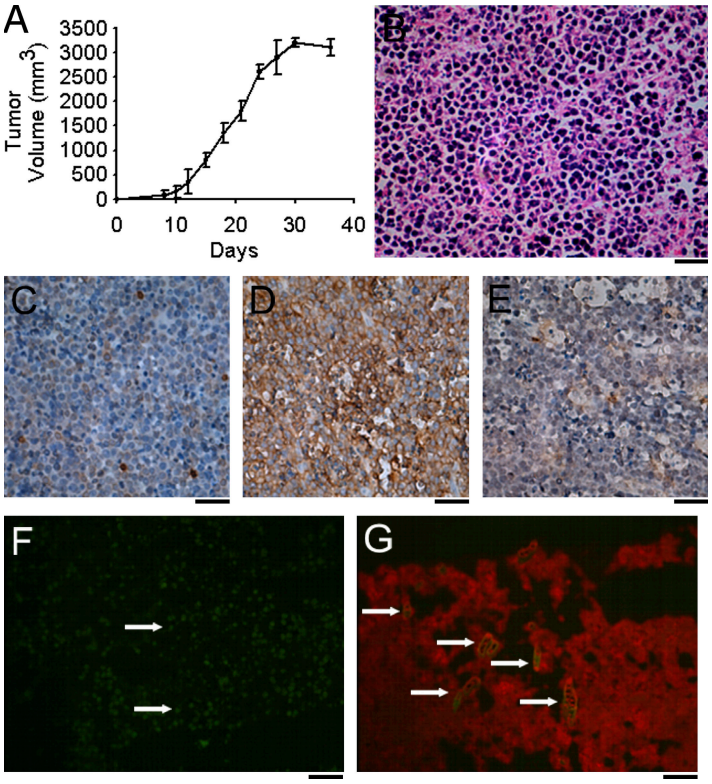

Figure S4. TA2 mice and transplantable B lymphoma cells were obtained from the Animal Experimental Center of Tianjin cancer institute. The transplantable B lymphoma cells were derived from spontaneous lymphoma in a TA2 mouse, remained stable past 43 generations by successful transplantations in TA2 mice over 4 yr, and developed rapidly growing and highly invasive metastases in 100% of mice. Mice were randomized when they were 4–6 wk of age and inoculated with 5×10^5 lymphoma cells into the inguinal groove muscle. The growth and dissemination of lymphoma were monitored weekly until mice were sacrificed. (A) Growth of lymphoma after inoculation. Data are presented as means ± SD of at least three experiments. (B) Representative histological images of lymphoma by hematoxylin and eosin staining. Immunohistochemical analysis was performed using antibodies against (C) CD3, (D) CD20, or (E) CD45RO. All images are hematoxylin counterstained. (F) ECs were isolated from the bone marrow of healthy TA2 mice, infected with ADV-GFP or ADV–Tim-3, and injected into the same site for lymphoma cell inoculation. The presence of adenoviral mutant–infected ECs in vivo was confirmed by tracking ADV-GFP. 1 d after the injection of ECs, the engrafted ECs scattered within the tumor (arrows). (G) 2 d after the injection of ECs, some microvessels formed by the engrafted ECs could be detected (arrows). Similar results were observed in two (A) or three (F and G) independent experiments. Bars, 20 µm.

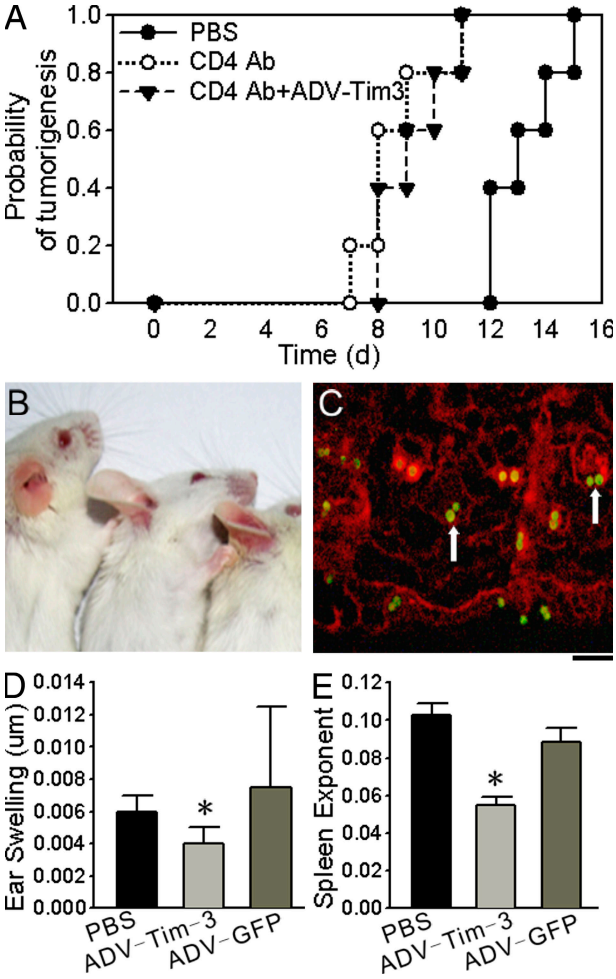

Figure S5. The onset of lymphoma in mice models pretreated with anti-CD4 blocking antibody and the effects of Tim-3–expressing ECs on the activation of T cell responses against DTH response. (A) BALB/c mice were inoculated with 5×10^5 A20 cells into the inguinal groove muscle. ECs were isolated and purified from the bone marrow of healthy BALB/c mice and infected with ADV–Tim-3 ($n = 16$) or PBS ($n = 8$). 5×10^4 ECs were injected into the same site for tumor inoculation 3, 6, and 9 d after tumor implantation. Half of the ADV–Tim-3–treated model mice ($n = 8$) were pretreated with CD4 blocking antibody (BioXcell) at a concentration of 20 mg/kg via intraperitoneal injection 3 d after lymphoma implantation. Cumulative probability of tumor onset was determined by the appearance of lymphoma in the inguinal groove muscle. (B) Injection of Tim-3–expressing ECs inhibited the activation of T cell responses against DTH response. The dinitrofluorobenzene (DNFB)-induced DTH test was performed. The sensitization phase was induced by topical application of 20 µl of 0.2% (vol/vol) DNFB in acetone onto the shaved abdomen on days 1 and 2 (total = 80 µg). To elicit DTH reactions, the animals were challenged by application of 20 µl of 0.2% DNFB in acetone (40 µg/ear, respectively) on the inner and outer surfaces of right ear 6 d after sensitization. 1 d after DTH, ear swelling and spleen exponent were determined. The difference of ear swelling was shown in ECs infected with ADV-GFP (right; $n = 6$), ADV–Tim-3 (middle; $n = 6$), or PBS (left; $n = 6$). (C) ECs were isolated from the bone marrow of healthy A20 mice and infected with PBS, ADV-GFP, or ADV–Tim-3. Approximately 4×10^4 ECs were injected into adjacent ear tissues on days 1, 2, and 3. The presence of adenoviral mutant–infected ECs in vivo was confirmed by tracking ADV-GFP (arrows). Bar, 20 µm. (D) The ear swelling was determined. Data are represented means ± SD of triplicates. *, $P < 0.01$. (E) The spleen exponent was calculated as spleen weight per 10 g of whole mouse weight. Data are represented means ± SD of triplicates.

Table S1. Fluctuation of lymphocyte subsets in umbilical vein T lymphocytes after stimulation of autologous ECs infected with various adenoviral mutants

Group	Lymphocyte[a]	CD4+ T Cell		CD8+ T Cell	
	$\times 10^6$	%	$\times 10^5$	%	$\times 10^5$
PBS	2.11 ± 0.35	42.43 ± 4.36	8.95 ± 1.35	28.71 ± 3.75	0.61 ± 0.09
ADV-GFP	2.17 ± 0.25	42.13 ± 3.11	9.14 ± 1.88	31.75 ± 2.96	0.69 ± 0.12
ADV-TT	2.68 ± 0.42	49.09 ± 4.03	13.16 ± 1.59	30.92 ± 3.06	0.83 ± 0.15
ADV-TT + ADV–Tim-3	2.1 ± 0.18	45.13 ± 5.02	9.48 ± 1.54	30.46 ± 4.03	0.64 ± 0.04
ADV-TT + ADV-GFP	2.7 ± 0.2	48.45 ± 2.17	13.08 ± 2.15	29.49 ± 3.52	0.8 ± 0.11

ADV-TT versus PBS or ADV-GFP in comparison to CD4+ cell numbers, $P < 0.05$; ADV–Tim-3 versus ADV-TT in comparison to CD4+ cell numbers, $P < 0.05$.
[a]Values represent means ± SE.

Table S2. Fluctuation of lymphocyte subsets in peripheral blood T lymphocytes after stimulation of autologous ECs infected with adenoviral mutants

Group	Lymphocyte[a]	CD3+CD4+ T Cell		CD3+CD8+ T Cell	
	$\times 10^5$	%	$\times 10^5$	%	$\times 10^5$
PBS	4.12 ± 0.36	40.39 ± 3.19	1.66 ± 0.39	16.8 ± 1.34	0.69 ± 0.12
Con peptide	4.09 ± 0.23	41.58 ± 2.67	1.7 ± 0.55	16.2 ± 1.67	0.66 ± 0.1
VEGFR-2 peptide	5.05 ± 0.17	37.65 ± 1.99	1.9 ± 0.67	16.75 ± 1.78	0.85 ± 0.23
VEGFR-2 peptide + ADV-GFP	4.85 ± 0.56	37.68 ± 1.67	1.83 ± 0.42	17.79 ± 0.97	0.86 ± 0.19
VEGFR-2 peptide + ADV–Tim-3	3.15 ± 0.43	33.43 ± 1.59	1.05 ± 0.32[b]	28.88 ± 2.18	0.91 ± 0.31

[a]Values represent means ± SE.
[b]VEGFR-2 peptide + ADV–Tim-3 versus VEGFR-2 peptide or VEGFR-2 peptide + ADV-GFP, $P < 0.05$.

Table S3. Tim-3–expressing ECs inhibit basal levels of Th1 cytokines in vivo

	TNF	IFN-γ	IL-5	IL-4	IL-2
	pg/ml	*pg/ml*	*pg/ml*	*pg/ml*	*pg/ml*
Blood					
PBS	116.9	0	0	0	0
ADV–Tim-3	88.2[a]	0	0	0	0
ADV-GFP	110.2	0	0	0	0
Spleen					
PBS	600.4	67.2	0	0	38.4
ADV–Tim-3	364.8[b]	48.9	0	0	34.8
ADV-GFP	536.2	60.8	0	0	52.2
Tumor					
PBS	160.4	108.7	0	0	28.5
ADV–Tim-3	114.9[b]	68.7[b]	0	0	27.3
ADV-GFP	170.5	125.6	0	0	27.8

All cytokine concentrations are given in pg/ml.
[a]ADV–Tim-3 versus PBS or ADV-GFP, $P < 0.05$.
[b]ADV–Tim-3 versus PBS or ADV-GFP, $P < 0.01$.