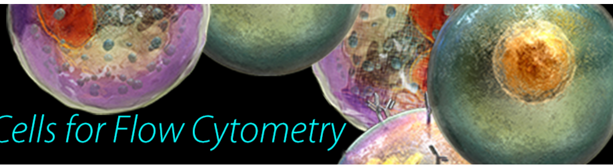

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Tim-3+ T-bet+ Tumor-Specific Th1 Cells Colocalize with and Inhibit Development and Growth of Murine Neoplasms[1]

William J. Simmons,[2]* Mythili Koneru,‡ Mani Mohindru,§ Rajan Thomas,† Scott Cutro,* Parul Singh,* Rosemarie H. DeKruyff,¶ Giorgio Inghirami,†‖ Anthony J. Coyle,# Byung S. Kim,§ and Nicholas M. Ponzio[3]*

Although T cells infiltrate many types of murine and human neoplasms, in many instances tumor-specific cytotoxicity is not observed. Strategies to stimulate CTL-mediated antitumor immunity have included in vitro stimulation and/or genetic engineering of T cells, followed by adoptive transfer into tumor-bearing hosts. In this model of B cell lymphoma in SJL/J mice, we used Tim-3$^+$ T-bet$^+$ Th1 cells to facilitate the development of tumor-specific CTL. Tumor-specific Th1 cell lines were polarized with IL-12 during in vitro stimulation and long term maintenance. As few as 5 million Tim-3$^+$ T-bet$^+$ Th1 cells enabled recipients to resist growth of malignant transplantable cells. In addition, similar numbers of Th1 cells injected into 2- to 3-mo-old mice inhibited development of the spontaneous primary lymphomas, which normally arise in 90% of aging mice. CFSE$^+$ Th1 cells colocalized with injected tumor cells in vivo and formed conjugates with the tumor cells within follicles, whereas in nontumor-challenged recipients the CFSE$^+$ Th1 cells localized only within the T cell zones of the spleen. These results provide evidence that adoptive immunotherapy with Tim-3$^+$ T-bet$^+$ tumor-specific Th1 cells can be used to induce host cytotoxic responses that inhibit the development and growth of neoplastic cells. *The Journal of Immunology,* **2005, 174: 1405–1415.**

Immunotherapy can be a beneficial option in the treatment of human cancers, especially those in which T cells appear to be activated, but an effective cytotoxic immune response is deficient. However, significant challenges have limited the exploration of this strategy both experimentally and clinically. Complications include selection for TCR specificities restricted to the tumor Ags, T cell expansion in response to tumor cell encounter, a tumor-specific immune response in vivo, long term survival of effector cells in vivo, and their localization to tumor cell foci (1).

Upon T cell activation and costimulation, either in vivo or in vitro, naive Th cells differentiate into distinct subsets that produce IFN-γ (Th1) or IL-4 and IL-5 (Th2). During activation of naive Th cells, addition of exogenous IL-4 induces Th2 differentiation, whereas addition of exogenous IL-12 potently promotes Th1 differentiation. Th cells are required for optimal primary expansion of CD8$^+$ T cells and their differentiation into CTL (2, 3). Despite the importance of Th cells for CTL development, the utility of adoptive transfer of Th cells to facilitate the production of tumor-specific CTL in vivo has received little attention.

Using a B cell lymphoma model in SJL/J mice, we investigated the efficacy of adoptive transfer of in vitro polarized, tumor-specific Th cell lines in vivo. The Th1 cell lines were CD4$^+$CD8$^-$Tim-3$^+$, used the transcription factor T-bet, but not GATA-3, and were TCR Vβ-chain restricted. Th cells proliferated upon stimulation by tumor and localized to the white pulp of the spleen after adoptive transfer, where they divided and formed conjugates with tumor cells. Th1, but not Th2, cells prevented the development of primary tumors and significantly reduced the growth of viable tumor cells in Th1-injected recipients. Together these results indicate that the adoptive transfer of tumor-specific Th1 cells is an effective and safe strategy in the prevention and treatment of spontaneous tumors.

Materials and Methods

Mice and tumor cells

SJL and C57BL/6 mice were purchased from The Jackson Laboratory. The in vitro cNJ117 tumor cell line (4) was derived from a spontaneous SJL tumor and maintained in culture with IMDMEM (Cellgro; Mediatech) supplemented with 10% FCS (Cellgro), OPI (Sigma-Aldrich), nonessential amino acids (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), penicillin/streptomycin, and 0.05 mM 2-ME. The in vivo germinal center-derived transplantable B lymphoma cell line, RCS-X, was originally propagated from a spontaneous SJL tumor and was maintained by weekly i.v. passage of 10^6–10^7 tumor cells (6–10 days posttumor transfer) into 6- to 12-wk-old syngeneic recipient mice.

RNA isolation, RT, and PCR

DNase I-treated total RNA was isolated from cells using TRIzol (Sigma-Aldrich). cDNA was synthesized using SuperScript (Invitrogen Life Technologies). The cDNAs were treated with RNase H for 20 min. Five microliters of cDNA was amplified with the GeneRacer RLM-RACE kit (Invitrogen Life Technologies). In the case of T-bet and Gata-3, thermocycling was performed for 5 min at 95°C, then 35 cycles of 15 s each at 95°C, 20 s at 58°C, and 30 s at 72°C. DNA was extended for 6 min at 72°C.

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To avoid linear phase amplification, PCR products were analyzed when 25, 30, 35, and 40 cycles were complete. Amplified DNA was analyzed by electrophoresis through 1.5% agarose containing 0.5 μg/ml ethidium bromide. Primer sequences were previously described (5).

Mixed lymphocyte responses

MLRs were performed as described previously (6). Tumor cells were irradiated (Mark I ^{137}Cs irradiator; JL Sheppard and Associates) before use as stimulator cells. In some cases Con A (Sigma-Aldrich) was used at a concentration of 10 μg/ml as a positive control stimulus for T cell proliferation.

Generation of cytotoxic effector cells

The use of IL-12 to stimulate the production of cytotoxic effector cells against the syngeneic SJL B cell lymphoma, RCS-X, was previously described (7). Briefly, SJL spleen cells (2×10^6/well) were cocultured with γ-RCS-X lymphoma cells (5×10^5/well) in multiple wells of 24-well plates in RPMI 1640 (Fisher Scientific) with or without 10 ng/ml IL-12. Cells from replicate wells were pooled, counted, and tested against ^{51}Cr-labeled target cells as described below. Dose-response and kinetic analyses revealed that 10 ng/ml IL-12 added at the initiation of a 4-day coculture of SJL spleen and γ-RCS-X lymphoma cells yielded optimal cytotoxic responses.

^{51}Cr release assay

A ^{51}Cr release assay was used to determine levels of cytotoxic activity as previously described (7, 8). cNJ117 was the CTL-susceptible SJL tumor cell line used as a target in the ^{51}Cr release assays. Isotope release from target cells incubated alone (for spontaneous release) and that from target cells incubated with 1% Nonidet P-40 (Sigma-Aldrich; for maximum release) were used in the calculation to determine the percent specific ^{51}Cr release due to CTL-mediated lysis of target cells.

Development of tumor-specific Th cell lines

Th cell lines were produced and used within a 12-mo period for the experiments presented in this paper. Naive SJL spleen cells were stimulated every 2 wk with γ-lymphoma cells and, in the case of Th1, but not Th2 lines, addition of IL-12 (10 ng/ml) in 24-well plates. The cells were expanded between stimulations by addition of human rIL-2 (100 U/ml) at regular intervals. The Th2 cell lines never received IL-12.

Flow cytometry

FITC- and/or PE-conjugated mAbs for surface markers were purchased from Caltag Laboratories or BD Pharmingen for one- or two-color phenotype analysis. Cells were lymphoid-gated and analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Intracellular cytokine staining was performed with the Cytofix/Cytoperm and the Golgi Stop kit (BD Pharmingen).

ELISA

Supernatants from triplicate cell cultures were harvested 24 h after stimulation and stored at -70°C until blind assay by the University of Maryland-Baltimore Cytokine Core Laboratory Biomedical Research Facility.

TCR Vβ-chain analysis of Th cells

Total RNA was reverse transcribed as previously described. The relative concentrations of cDNA were estimated based on the level of β-actin amplification (35 cycles) by PCR. Each Vβ was assessed by PCR analysis using a sense sequence primer of the individual Vβs (Vβ1, -2, -3, -4, -5, -6, -7, -10, -14, -15, -16, -17, -18, and -19) and a common antisense primer of downstream Cβ as previously described (9). The TCR Vβ chain oligonucleotide primer sequences are shown in Table II.

In vivo RCS growth assay

Cells from the in vivo transplantable lymphoma cell line, RCS-X, were purified by depleting T cells with a mAb mixture (anti-TCR, CD4, and CD8) at 4°C for 30 min, followed by lysis with low toxicity rabbit complement at 37°C for 45 min as previously described (10, 11). SJL/J recipients were injected i.v. with 2×10^4 purified tumor cells. When control mice showed palpable enlargement of lymph nodes and spleen (typically 9–12 days posttransfer), all mice were killed, and the weight of their lymphoid tissues was expressed as a percentage of total body weight as a measure of tumor burden. To enumerate tumor cell content, cell suspensions prepared from the lymphoid tissues were also analyzed by FACS based on the expression of cell surface markers. Because H-2D^s molecules are expressed on normal SJL B cells, but not on the transplantable RCS-X tumor cells used in this study (12), we were able to distinguish these two populations by FACS analysis.

Detection of primary RCS development in vivo

Mice in long term observational studies received weekly examinations, including palpation of the secondary lymphoid organs. Tissues harvested from mice were immediately fixed in Telly's or formalin fixative and embedded, then sectioned and stained as previously described (13).

CFSE labeling of cells

CFSE (7 mM; Molecular Probes) was diluted 1/1000 and added to cells at 37°C for 1 h. Cell suspensions were washed at 4°C with PBS and 0.2% FCS twice and resuspended in complete medium for culture or in PBS for injection.

Statistics

Statistical significance was calculated by Student's *t* test using Cricket Statistics (Cricket Software) and PRISM (GraphPad) software.

Results

NFAT-dependent division and IL-12-dependent cytotoxicity of T cells

Activated T cells are present in a number of mouse and human neoplasms (14), yet cytotoxicity to tumor targets is not readily observed. To better understand the T cell response to tumor cells, we cultured naive SJL spleen cells with irradiated (γ-) in vivo transplantable lymphoma cells at a 4:1 responder to stimulator cell ratio for 4 days (Fig. 1*a*). The tumor-induced T cell proliferation was blocked in a dose-dependent fashion by inclusion of CD4 mAb, whereas Con A proliferation was slightly blocked at only the highest Ab concentration (Fig. 1*b*). When CFSE-labeled T cells were cultured with tumor cells, similar T cell proliferation was observed by FACS analysis (data not shown). T cell proliferation to nominal Ag presentation is IL-2 dependent, and NFAT is important for the production of IL-2 (15). Therefore, we also determined whether the addition of cyclosporin A (CSA),[4] an inhibitor of NFAT, to cultures of tumor-stimulated spleen cells blocked T cell proliferation (16). As shown in Fig. 1*c*, addition of CSA significantly blocked the tumor-induced T cell response ($p < 0.001$) as well as the Con A response (Fig. 1*d*), which was expected because Con A-responsive T cells use NFAT to induce IL-2 production.

Despite the induction of proliferation by tumor cells (Fig. 1, *a* and *c*), tumor-specific cytotoxicity did not develop in 4-day cocultures of naive spleen cells and tumor cells (Fig. 1*e*, *lower curve*). Cytotoxicity was obtained, however, from similar stimulation of naive allogeneic (C57BL/6) spleen cells with tumor cells (data not shown). Previous studies indicated that the in vitro SJL T cell response to tumor in the presence of exogenous IL-12 led to the development of tumor-specific cytotoxicity (7), consistent with the reported effects of IL-12 during nominal Ag presentation (17). Moreover, when IL-12 was included in cultures that contained tumor and naive spleen cells, cytotoxicity to tumor targets was observed (Fig. 1*e*, *upper curve*) and was induced using a broad range of responder to stimulator cell ratios, from 4:1 (Fig. 1*c*) to 40:1 (not shown). However, when SJL mice were injected with 2 μg of IL-12 for 5 days, then challenged with 2×10^4 viable transplantable tumor cells, tumor growth was similar to that in vehicle- and IL-2-injected controls (not shown). This result, however, may have been influenced by the morbidity induced by IL-12 in these mice despite use of an IL-12 dose that was in concordance with

[4] Abbreviations used in this paper: CSA, cyclosporin A; γ-, irradiated; PNA, peanut agglutinin.

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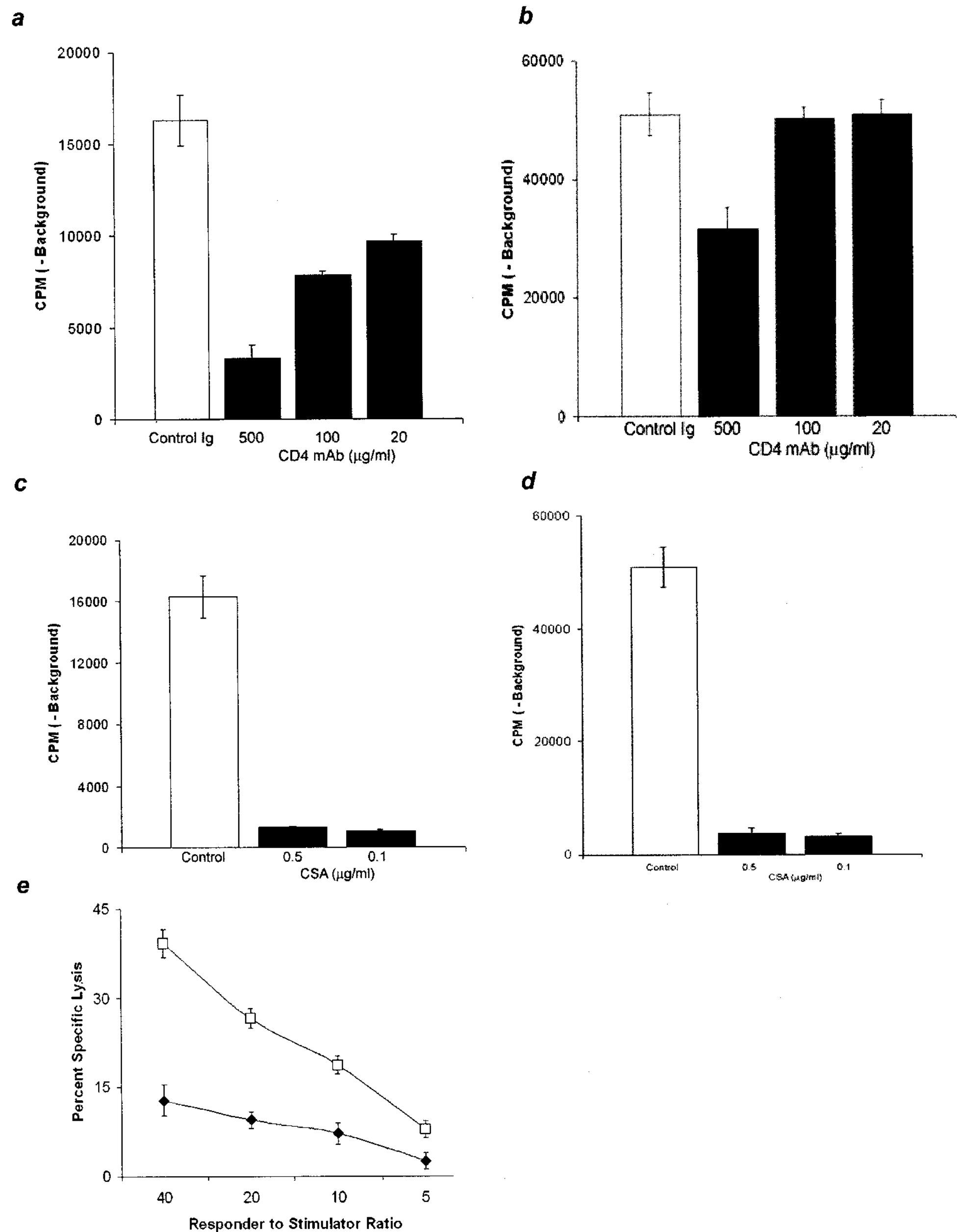

FIGURE 1. Characterization of the T cell response to tumor cells. CD4 T cell proliferation was measured in: *a*) 4-day cultures of SJL spleen cells and irradiated in vivo tumor cells (10,000 rad), and *b*) Con A-stimulated spleen cells in the presence of CD4 mAb (■) or isotype-matched control Ig (□). The cultures were pulsed with [^{3}H]thymidine 18 h before harvest. NFAT dependence was determined by inclusion of CSA (■) or control ethanol (□) in 5-day cultures of irradiated tumor cells and naive spleen cells (*c*) or Con A-stimulated spleen cells (*d*). *e*, Inclusion of IL-12 stimulates the development of cytotoxic effector cells. Spleen cells were cocultured with γ-tumor cells at a responder to stimulator cell ratio of 4:1 in the presence (□) or the absence (♦) of 10 ng/ml IL-12 for 4 days. Resulting effector cells were harvested, then cocultured with radiolabeled targets for 4 h at the indicated E:T cell ratios (abscissa).

other experiments (18, 19). Therefore, it was compelling to design a strategy that would exploit this IL-12-induced cytotoxicity to safely obtain antitumor efficacy in vivo.

Characterization of Th cell lines

To produce Th cell lines, cocultures of SJL spleen and γ-lymphoma cells were established. For Th1 cell lines, cultures were stimulated with γ-tumor cells every 14 days in the presence of exogenous IL-12, then expanded with IL-2, typically 4–5 days after stimulation. Stimulation/expansion was repeated every 2 wk. A similar protocol was used to develop Th2 cell lines, but without inclusion of exogenous IL-12.

T cell lines were assayed for surface markers by FACS analysis (Table I) to reveal their Th cell lineage (TCR $\alpha\beta^+$,$CD3^+$,$CD4^+$, but $B220^-$,$CD8^-$). All cell lines were IL-$2R^+$ (CD25), suggesting the importance of IL-2 for their continued growth. T1/ST2, a putative Th2 cell marker (20, 21), was detectable on Th2, but not Th1, cell lines (Table I). Tim-3 (22) also distinguished the Th cell subsets, being expressed on Th1 cells (Fig. 2*a*, *columns 3* and *4*), but not on Th2 cells (Fig. 2*a*, *columns 1* and *2*).

Another criterion we used to distinguish Th cell lines was production of IFN-γ (Th1) or IL-4 (Th2), respectively. Th2 cells produced intracellular IL-4 (Fig. 2*b*), whereas staining of T cell lines polarized in the presence of IL-12 with anti-IL-4 mAb did not differ significantly from that of the isotype control. Fig. 2*b* also shows the results obtained from intracellular staining with anti-IFN-γ mAb and control isotype-matched Ig. The mean fluorescence intensity of Th cell lines obtained from cultures to which exogenous IL-12 was added during polarization (Th1) was significantly higher than isotype control levels. However, Th cell lines obtained under Th2-polarizing conditions (without IL-12) did not contain IFN-γ. We also determined the levels of secreted IL-4 and IFN-γ in the culture supernatants of these cell lines. T cell lines were stimulated with γ-tumor cells for 24 h, and the supernatants from these cultures were analyzed in cytokine-specific ELISA. As shown in Fig. 2*c*, Th1-polarized cell lines secreted IFN-γ, but no

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detectable amounts of IL-4. In contrast, the Th2 cell lines polarized without IL-12 secreted IL-4, but little IFN-γ. Importantly, as shown in Fig. 2*c*, once polarized to a Th1 phenotype, stimulation with γ-tumor cells alone was sufficient to induce IFN-γ secretion, indicating that cytokine production by the polarized, tumor-responsive Th1 cells was not IL-12 dependent.

Recent reports suggested that Th1 and Th2 cells use different transcription factors: Th2 cells use the transcription factor GATA-3 (23), and Th1 T cells use T-bet (24). Fig. 2*d* shows that mRNA transcripts for T-bet, but not GATA-3, were produced by Th1 cell lines, whereas GATA-3, but not T-bet, was detectable in Th2-polarized T cells. These results indicate that tumor-stimulated Th1 cells, like Th1 cells responding to nominal Ags, use different transcription factors than those polarized under Th2 conditions and thus produce different cytokines.

Due to recognition of mouse mammary tumor virus-encoded vSAg in SJL lymphoma cells, there is skewing of TCR Vβ chains expressed on responding SJL T cells (13, 25). Therefore, we determined whether the TCR Vβ-chains of the tumor-responsive Th cell lines were skewed toward a particular Vβ. cDNA from the Th cell lines was included in a PCR using primers for every TCR β-chain represented in the SJL T cell repertoire, as well as the negative control, Vβ5. The oligonucleotide primer sequences used for these assays are shown in Table II. All Th1 and Th2 cell lines were skewed with respect to their TCR Vβ specificity (Fig. 2*e*), and the degree of Vβ clonality appeared to improve with increasing cycles of stimulation and expansion of the Th cell lines (W. J. Simmons and N. M. Ponzio, unpublished observations).

Specificity of Th cell lines for tumor

To determine the specificity of the Th cell lines, CFSE-labeled Th cell lines were incubated with and without γ-tumor cells. Fig. 3*a* illustrates the vigorous proliferation (indicated by peaks with diminished immunofluorescence intensity) of the Th cell lines in response to both in vitro and in vivo transplantable lymphoma cells. By day 4 of culture, the mean fluorescence intensity of Th cells dropped by as much as 6-fold after stimulation by in vivo tumor cells. This proliferation was blocked when MHC II (I-A^s) mAb was added to the cultures (data not shown), demonstrating the presentation of mouse mammary tumor virus vSAg by these molecules on the tumor cells (26). In contrast, 4-day cocultures of CFSE-labeled Th cell lines and irradiated allogeneic C57BL/6 spleen cells revealed minimal cell proliferation (data not shown), underscoring the tumor cell specificity of the Th cell lines.

Table I. *FACS analysis of cell surface markers on Th cell lines*

	T Cell Lines			
	Th2 cell lines		Th1 cell lines	
Markers	SB.10	WB4	A3	A312
TCR$\alpha\beta$	+	+	+	+
CD3	+	+	+	+
CD4	+	+	+	+
CD25	+	+	+	+
CD30	+	+	+	+
CD69	+	+	+	+
CD8	–	–	–	–
B220	–	–	–	–
T1/ST2	+	+	–	–
Tim-3	–	–	+	+

Th1 cells eliminate the need for IL-12 for CTL development

The development of cytotoxicity by spleen cells against tumor targets was IL-12 dependent, indicating that the Th1-polarizing effect of IL-12 mediated the production of IFN-γ (Fig. 2, *b* and *c*) and possibly other cytokines needed for CTL development. Addition of either exogenous IL-12 or Th1 cell lines resulted in the generation of tumor-specific CTL (Fig. 3*b*, *upper two curves*). However, in cocultures without IL-12 or to which Th2 cells were added, no increase in specific lysis was observed (Fig. 3*b*, *lower two curves*). This indicated that tumor-specific Th1, but not Th2, cell lines could replace the need for exogenous IL-12 in vitro. To rule out the possibility that the Th1 cell lines were directly cytotoxic to tumor targets, we cultured tumor-activated Th1 or Th2 cell lines with ^{51}Cr-labeled tumor target cells. The data presented in Fig. 3*c* show that neither Th1 nor Th2 cell lines directly lysed tumor targets, although lysis was observed by CTL from control cocultures of naive SJL spleen and γ-lymphoma cells containing IL-12.

Efficacy and localization of Th1 cells in vivo

Groups of 12-wk-old SJL mice were given a single injection of 5×10^6 Tim-3$^+$ Th1 cells, staggered such that each group received a single injection of cells 7 days apart until the last group was injected on day 21. One week after the last group received Th1 cells, their spleen cells were placed into cocultures with γ-tumor cells without IL-12. Spleen cells from age-matched, naive SJL donors were also cultured with γ-tumor cells with or without IL-12 as a control. On the fourth day of culture, cells were harvested and used as effector cells in a ^{51}Cr release assay against tumor targets. Fig. 3*d* shows that Th1 cells resident in vivo for 7 or 14 days supported the development of tumor-specific cytotoxic effector cells when restimulated in vitro with γ-tumor cells in the absence of IL-12, similar to values from control tumor-stimulated naive spleen cell cultures to which exogenous IL-12 had been added directly. However, levels of cytotoxicity in spleens from mice in which Th1 cells were resident for 3 or 4 wk were not elevated above those in naive spleen cells stimulated with tumor cells in the absence of IL-12.

To determine whether Th cell lines could influence the in vivo growth of tumors, we injected 8-wk-old SJL mice with 5×10^6 tumor-specific Th1 or Th2 cell lines and 10 days later challenged them with 2×10^4 purified (T cell-depleted) viable malignant lymphoma cells. When vehicle-injected control mice showed tumor growth, all mice were killed. Th1-injected mice had significantly less growth ($p < 0.001$) in both lymph nodes (not shown) and spleens (Fig. 4*a*) compared with control vehicle-injected mice. FACS analysis of cells from lymphoid tissues of these mice showed that the reduction in spleen weight also correlated with a decrease in the number of tumor cells. The absolute number of tumor cells in the spleen (94.8×10^6) was significantly reduced ($p < 0.01$) in Th1-injected mice compared with control vehicle-injected mice (271.2×10^6). Interestingly, in mice injected with tumor-specific Th2 cell lines, growth of tumor in the lymph nodes ($p = 0.01$), but not in the spleens ($p = 0.4$), was enhanced (Fig. 4*a*).

As an additional specificity control, we compared the ability of Ag nonspecific Th1 cells to influence tumor growth in vivo. Growth of B lymphoma cells in mice injected with cells from a SJL/J Th1 clone specific for Theiler's virus peptide (9) was no different from that measured in control mice challenged with tumor cells alone. In this experiment the lymph nodes and spleens of tumor-injected control mice ($n = 6$) were 3.57 and 3.50% of total body weight, respectively, and the lymph nodes and spleens of Ag-nonspecific, Th1-injected, tumor-challenged mice ($n = 8$) were 4.71 and 4.25% of total body weight, respectively. Therefore,

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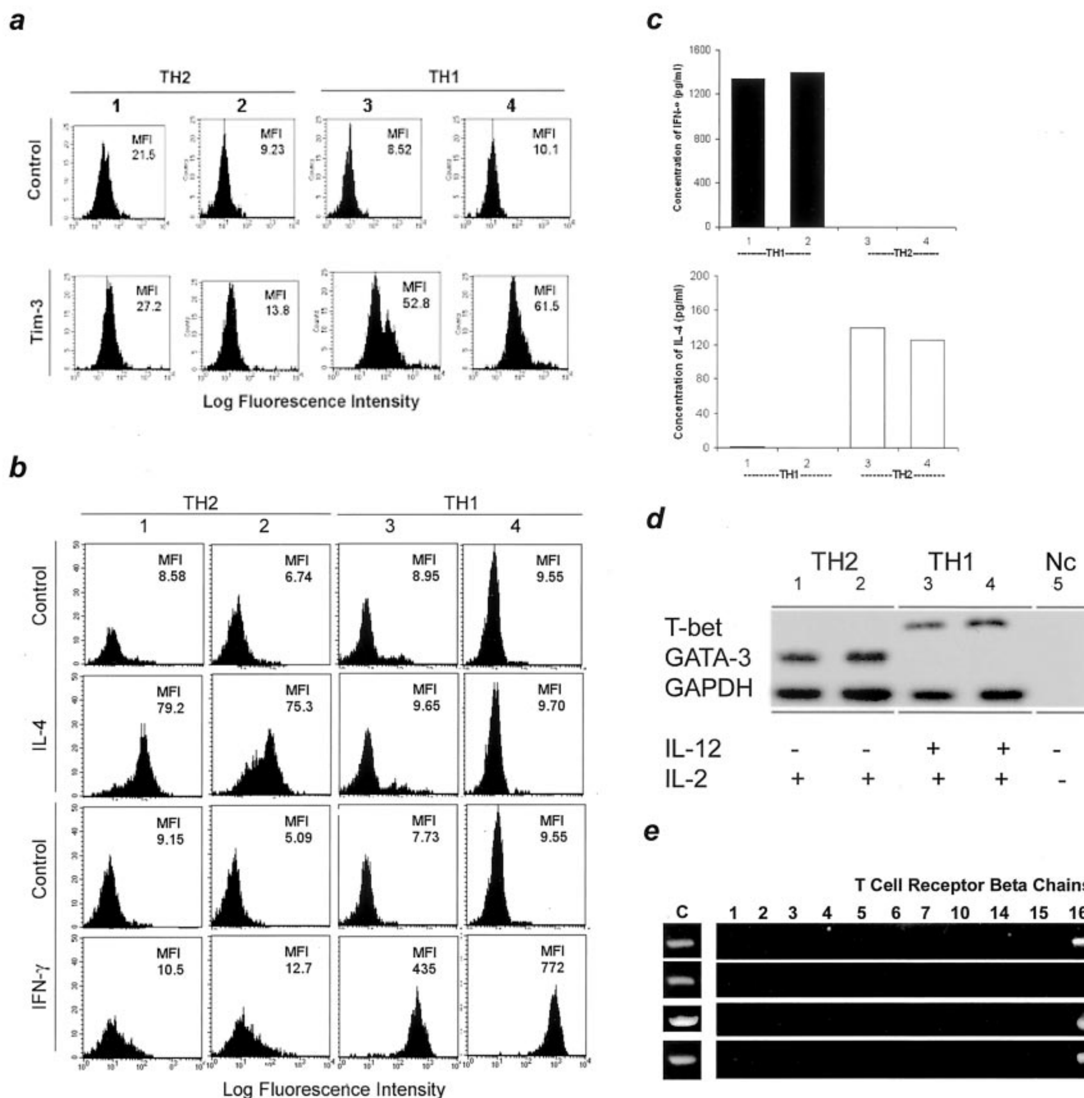

FIGURE 2. Characterization of tumor-reactive Th cell lines. *a*, Th cells were analyzed by FACS after incubation with purified Tim-3 mAb compared with isotype-matched controls, followed by incubation with a fluorescence-labeled secondary Ab. *Columns 1–4* are Th cell lines SB.10 (Th2), WB4 (Th2), A3 (Th1), and A3.12 (Th1), respectively. *b*, Production of IL-4 and IFN-γ was determined by intracellular staining with fluorescence-labeled mAb for each cytokine after permeabilization of the cell membranes. Isotype control mAb staining for each cell line is shown. *Columns 1–4* are lines WB4 (Th2), SB.10 (Th2), C2 (Th1), and C6 (Th1), respectively. *c*, IL-4 (□) and IFN-γ (■) secretion by Th cell lines was determined by stimulating with tumor for 24 h (4:1 responder to stimulator cell ratio). In the cases of Th1 cell lines C2 (*column 1*) and C6 (*column 2*), exogenous IL-12 was not added to the culture medium to ensure that cytokine production was not dependent on the presence of Il-12. Likewise, Th2 cell lines WB4 (*column 3*) and SB.10 (*column 4*) did not receive supplementation with IL-12. Background values were obtained from medium alone (IFN-γ, 19 pg/ml; IL-4, 1 pg/ml). *d*, Total mRNA was isolated from various Th cell lines (WB4 (Th2), SB.10 (Th2), A3.12 (Th1), and A3 (Th1); *columns 1–4*, respectively). RNA was reverse transcribed to cDNA, and PCR was performed using the same volume of cDNA for primers specific for T-bet and GATA-3. GAPDH was amplified for comparison. PCR samples without cDNA were invariably negative (*lane 5*). *e*, mRNA was obtained from Th lines 3 days poststimulation with tumor. cDNA was synthesized from each RNA preparation and then amplified by PCR using an individual Vβ (Vβ1, -2, -3, -4, -5, -6, -7, -10, -14, -15, -16, -17, -18, and -19) sense primer and a common Cβ antisense downstream primer. Vβ5, a TCR β-chain that is deleted in the genome of SJL mice, was used as a negative control.

the inhibition of lymphoma growth shown in Fig. 4*a* was both Th1 dependent and tumor Ag specific.

Tumor-free, 2- to 3-mo-old SJL mice were also given a single injection of 5–10 × 10^6 Th cell lines and monitored for another year, during which time 86% of untreated SJL mice develop spontaneous primary lymphomas (27). As the survival curves in Fig. 4*b* show, injection of Th1 cell lines abrogated the development of primary lymphomas, because only 7% of mice injected with Th1 cell lines ($n = 20$) developed primary tumors at 14 mo of age vs 90% of age-matched control mice of the same age ($n = 15$). The frequency and time of onset of primary lymphomas in normal SJL mice are similar to those in previous studies (26) in which greater numbers of mice were followed ($n = 170$). The development of primary tumors was significantly inhibited in Th1-injected mice ($p < 0.001$), whereas 100% of Th2 cell line-injected mice developed spontaneous primary tumors at a somewhat accelerated frequency compared with control mice.

Additional histological analysis of the spleens of these mice revealed normal-appearing architecture with defined regions of primary B cell follicles and T cell areas as well as red pulp (Fig. 4*c*, *A*). A higher power view (×40) showed normal-appearing small lymphocytes throughout (Fig. 4*c*, *B*). Mice injected with vehicle alone, however, lacked normal splenic architecture and contained numerous neoplastic lymphoblastoid cells (Fig. 4*c*, *I* and

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Table II. *TCR Vβ chain oligonucleotide primer sequences*

TCR β-Chain	Primer Sequences (5′ to 3′)
Vβ1	CCCAGACAGCTCCAAGCTACTTT
Vβ2	CCACACGGGTCACTGATACGGAGC
Vβ3	CCTTCAAACTCACCTTGCAGC
Vβ4	TCAAAGAAAAACCATTTAGAC
Vβ5	TCTGGGGTTGTCCAGTCTCCA
Vβ6	GAGAAGAAGTCATCTTTTTCT
Vβ7	AAGAAGCGGGAGCATTTCTCC
Vβ10	TCAGATAAAGCTCATTTGAAT
Vβ14	TCGGTGGTGCAACTGAACCTC
Vβ15	CCCAACTTATCCTTTTCATCT
Vβ16	CCAGATGGTTCATATTTCACT
Vβ17	TACCTGGTCAAAGAGAAAGGA
Vβ18	CATCTGTCAAAGTGGCACTTCA
Vβ19	AGACATCTGGTCAAAGGAAAAG
Cβ upstream	ATGGCTCAAACAAGGAGACCTTGG
Cβ downstream	GACAGGTTTGGGTGAGCCCTCTGGCCA

J). Mice that received 5×10^6 Th2 cell clones exhibited histology (not shown) resembling that of the vehicle-injected mice bearing primary tumors. These findings were confirmed by more definitive histochemical studies to identify the lymphoma cells by their expression of B220 and receptors for peanut agglutinin (PNA).

Th1-injected mice contained clearly stained $B220^+$ cells that were mostly restricted to the white pulp of the spleen (Fig. 4*c*, *C* and *D*). In contrast, vehicle- or Th2-injected mice contained $B220^+$ cells that were present, without organization, throughout the white pulp (Fig. 4*c*, *K* and *L*). In addition, $B220^+$ cells of Th1-injected mice appeared to bind PNA only at germinal centers. Tumor-bearing mice that received Th2 cell lines contained PNA-binding cells that were not localized to germinal centers, but dispersed throughout the spleen (data not shown) like those typical of SJL mice with primary tumors (6).

These results suggested that Th1 cells migrated to neoplastic sites to prevent tumor growth. However, to specifically determine Th cell localization, young SJL mice were injected with 5×10^6 CFSE-labeled Th1 cells. Three months later, one group was challenged with 5×10^4 viable tumor cells; 48 h later the mice were killed, and their spleens were frozen, sectioned, and counterstained with PE-conjugated anti-CD19 mAb. Fig. 5 shows photomicrographs of representative spleen sections from both tumor-challenged and nonchallenged Th1 cell-injected mice. In mice that were not injected with tumor cells, $CFSE^+$ Th1 cells localized to the white pulp of the spleen around, but not within, $CD19^+$ B cells areas. In contrast, in the tumor-challenged mice, $CFSE^+$ Th1 cells migrated not only to the T cell zones, but also into B cell areas, forming conjugates with $CD19^+$ cells. Presumably, the interactions reflect immunological synapses between the tumor-specific $CFSE^+$ Th1 cells and $CD19^+$ tumor cells. However, we cannot rule out the possibility that the interaction of tumor cells and Th1 cells within follicles promotes interactions between $CFSE^+$ Th1 cells and nonmalignant $CD19^+$ B cells that are resident within the follicles. Quantitation of the interfaces between Th1 and $CD19^+$ cells (see *inset* in Fig. 5*h*) are shown in the graphs at the *bottom* of the figure.

Discussion

Tumor immunity is dependent upon recognition and eradication of tumor cells by immune effector cells. Although tumor Ag recognition is necessary to elicit immunity, it alone is insufficient for tumor eradication; tumor-specific CTLs need to develop and expand, mature into effector cells, localize to tumor cells, and destroy them. This potential makes T cells crucial members of the adaptive immune response that may dramatically influence the development and/or growth of neoplastic cells.

SJL mice reliably develop spontaneous B cell lymphomas at a very high incidence (Fig. 4*b*), and although these tumors contain T cells, like many human tumors, cytotoxicity is not observed (7, 27). Prior experiments with this model revealed that in vitro cytotoxicity was IL-12 dependent (7). Although IL-12, IL-18, and IL-23 have been implicated in Th1 and CD8 T cell development, IL-12 is known to induce a Th1 phenotype (28) (Fig. 1*c*). However, IL-18 alone does not induce a Th1 response and, in the absence of IL-12, may stimulate Th2 responses (29). It is therefore possible that IL-18 and IL-23 could enhance, but may be unnecessary for, the Th1-mediated tumor immunity we observed (Figs. 1*c* and 4, *a* and *b*).

IL-12 immunotherapy enhances IFN-γ production, thereby inducing the transcription of cytotoxic granule-associated and adhesion molecules of CTL and NK cells (17, 30). Consistent with these observations, our results show that effector T cell development and lysis of tumor targets were influenced by the addition of exogenous IL-12 (Fig. 1*c*). However, treatment of tumor-injected SJL mice with IL-12 failed to inhibit tumor growth, perhaps due to its known toxicity (31, 32). On the basis of these results, we opted not to pursue the use of IL-12 in vivo, by either ectopic gene expression or pharmaceutical agent, to inhibit tumor growth. Rather, we developed a strategy, using IL-12 in vitro, to develop tumor-responsive Th cell lines that could influence in vivo tumor growth.

A major aim for T cell immunotherapy is to optimize T cell responses to the target Ag-bearing cells. Therefore, we selected for T cells responsive to tumor cells in vitro and determined that the Th cells could proliferate to tumor cells (but not allogeneic cells) in a CD4- and MHC II-dependent fashion (Figs. 1*a* and 3*a*). Tumor-induced T cell proliferation was also NFAT and IL-2 dependent (Fig. 1*c*). Our inclusion of IL-12 in the cocultures of naive SJL spleen and γ-lymphoma cells resulted in the production of tumor-responsive T cells that differentiated along a Th1 pathway. Tim-3 has been identified as a putative marker for Th1, but not Th2, cells. Moreover, agonism of Tim-3 by a mAb significantly exacerbated Th1-mediated pathology (22, 33), suggesting a potential negative regulatory role for endogenous Tim3-Tim3 ligand in vivo (V. K. Kuchroo, unpublished observations). Indeed, our data support the differential expression of this marker by Th subsets (Fig. 2*a*). In concordance with earlier reports by others (21), T1/ST2 also distinguished Th cells. Therefore, based solely on the expression of TCR, CD4, CD25, and Tim-3, but not T1/ST2, the Th cell lines polarized with IL-12 were of Th1 lineage (Table I).

Th1 cell subsets are also differentiated by the production of IFN-γ or IL-4. Our data clearly show that only Th1 cell lines contained intracellular IFN-γ, but not IL-4 (Fig. 2*b*), and the same differential pattern was observed in the Th cell supernatants (Fig. 2*c*). Interestingly, T-bet was identified as a Th1 cell-specific factor that induces the production of IFN-γ by developing Th1 cells (24). In addition to its role in inducing the expression of IFN-γ, T-bet seems to induce the expression of IL-12R β2 subunit (34, 35) and is expressed by naive T cells after Stat-1-mediated IFN-γ signaling (34, 36). Consistent with these observations, Th1, but not Th2, cells expressed T-bet (Fig. 2*d*). Therefore, on the basis of cell surface markers, cytokine patterns, and the use of transcription factors, the tumor-specific Th cells polarized with IL-12 were Th1.

Th1 cell specificity and responsiveness are required for effective T cell immunity. It was probable, using our strategy of repeated stimulation and expansion, that any tumor-nonspecific T cells present in the culture would not survive, but we could not rule out the bystander activation of such T cells. However, because the

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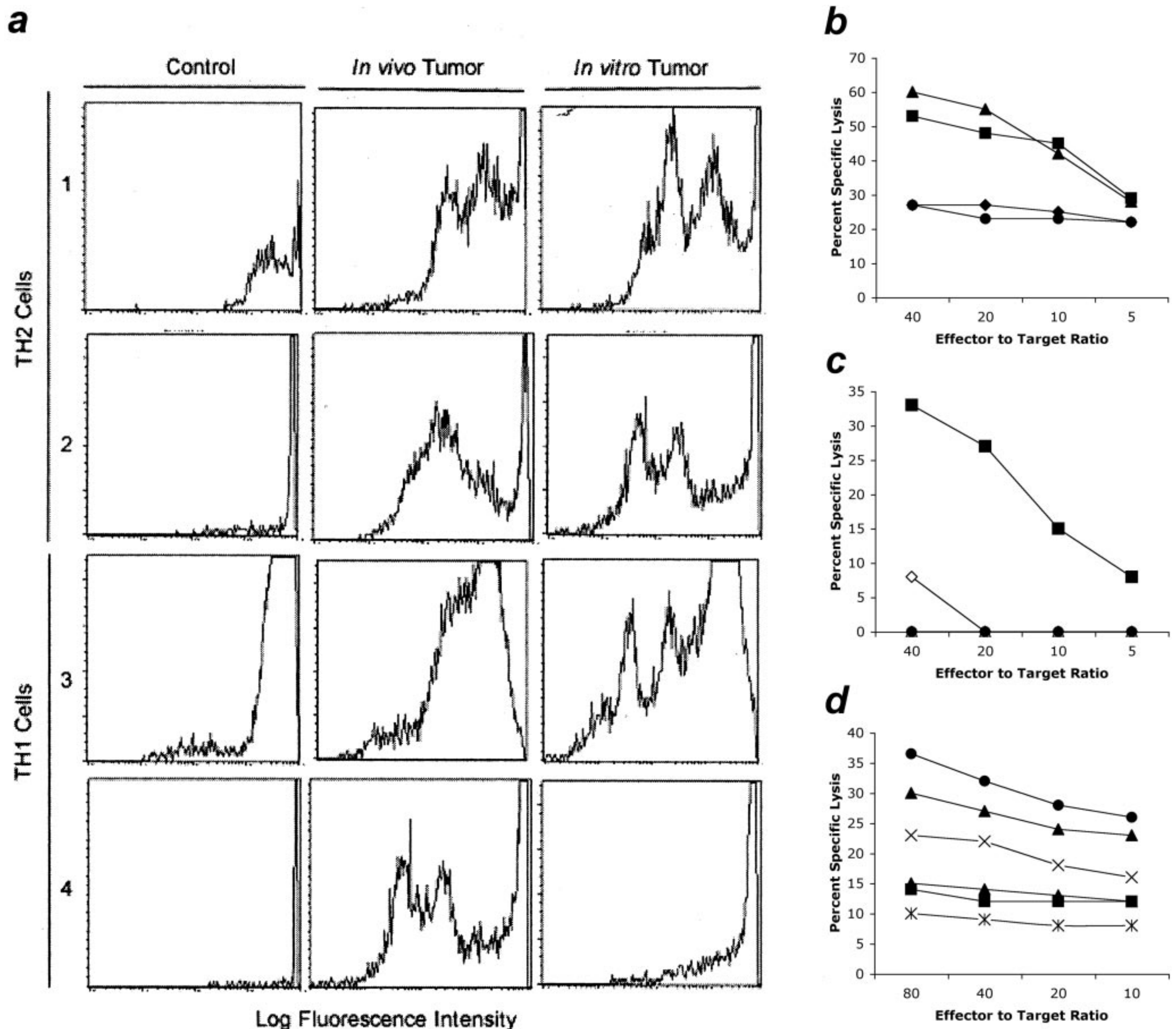

FIGURE 3. Function and specificity of Th1 cell lines. *a,* Th cell proliferation in response to in vivo (RCSX) and in vitro (cNJ117) tumor was determined by addition of CFSE-labeled Th cells to tumor cells (4:1 and 10:1 responder to stimulator cell ratios, respectively). Th1 cocultures contained 10 ng/ml exogenous IL-12. On day 5 the cells were washed and analyzed by FACS for CFSE content. *Rows 1* and *2* are Th cell lines SB.10 and WB4; *rows 3* and *4* are Th cell lines C2 and C6. *b*, Cultures of naive spleen cells and tumor cells (♦) were established to which IL-12 (■), Th1 (▲), or Th2 (x) cell lines were added. After 5 days of culture, cells were harvested and used as effector cells vs ^{51}Cr-labeled tumor target cells at various E:T cell ratios as indicated. *c*, Th1 (♦) and Th2 (●) cell lines failed to lyse tumor targets when stimulated directly with γ-tumor cells. Cocultures of naive SJL spleen cells and γ-tumor cells without (◇) and with (■) inclusion of IL-12 are shown as negative and positive controls, respectively. *d*, Spleen cells from SJL mice injected with Th1 cell lines for 1 or 2 wk, but not 3 or 4 wk, possess the ability to develop cytotoxicity against tumor target cells. Groups of 8-wk-old SJL mice were injected i.v. with a single dose of 5×10^6 Th1 cells at 0, 1, 2, and 3 wk (▲, ×, ♦, ■, respectively). Cocultures of a naive SJL spleen and γ-tumor cells without (⁎) and with (●) inclusion of IL-12 are the negative and positive controls, respectively. After 4 wk, all mice were killed, and their spleen cells were stimulated in vitro with γ-tumor cells in the absence of exogenous IL-12. After 5 days of culture, cells were harvested and used as effector cells vs tumor targets at various E:T cell ratios as indicated.

tumor cells express a vSAg encoded by an endogenous retrovirus, one would expect that the tumor-specific Th cells would express the same TCR Vβ (13). All Th cell lines were restricted with respect to their Vβ-chains; the predominant chain was Vβ16 (Fig. 2*e*). These data coupled with the observation that Th cells were unresponsive to allogeneic stimulation (data not shown) suggest that the Th1 cell lines were tumor specific.

However, the real measure of Th cell specificity and responsiveness is tumor-specific immunity. It is known that optimal CTL responses require a variety of cytokines, including IFN-γ (37). Therefore, we determined whether Th1 cell lines could replace the need for addition of exogenous IL-12, by virtue of their direct IFN-γ production (Fig. 2, *b* and *c*). It was possible that the Th1 cells produced not only IFN-γ, but also IL-18 and IL-23, which promoted the development of CTL. Alternatively, it was also possible that the tumor-activated Th1 clones were directly cytotoxic to tumor targets, because CD4 T cells may, under certain conditions, be cytotoxic effector cells themselves (38). Our data indicate that Th1 cells did not lyse tumor targets (Fig. 3*c*). Therefore, the most likely explanation for the appearance of cytotoxicity in cocultures of spleen and tumor cells with IL-12 is the development of tumor-specific Th1 cells that produce IFN-γ and perhaps other cytokines that lead to the

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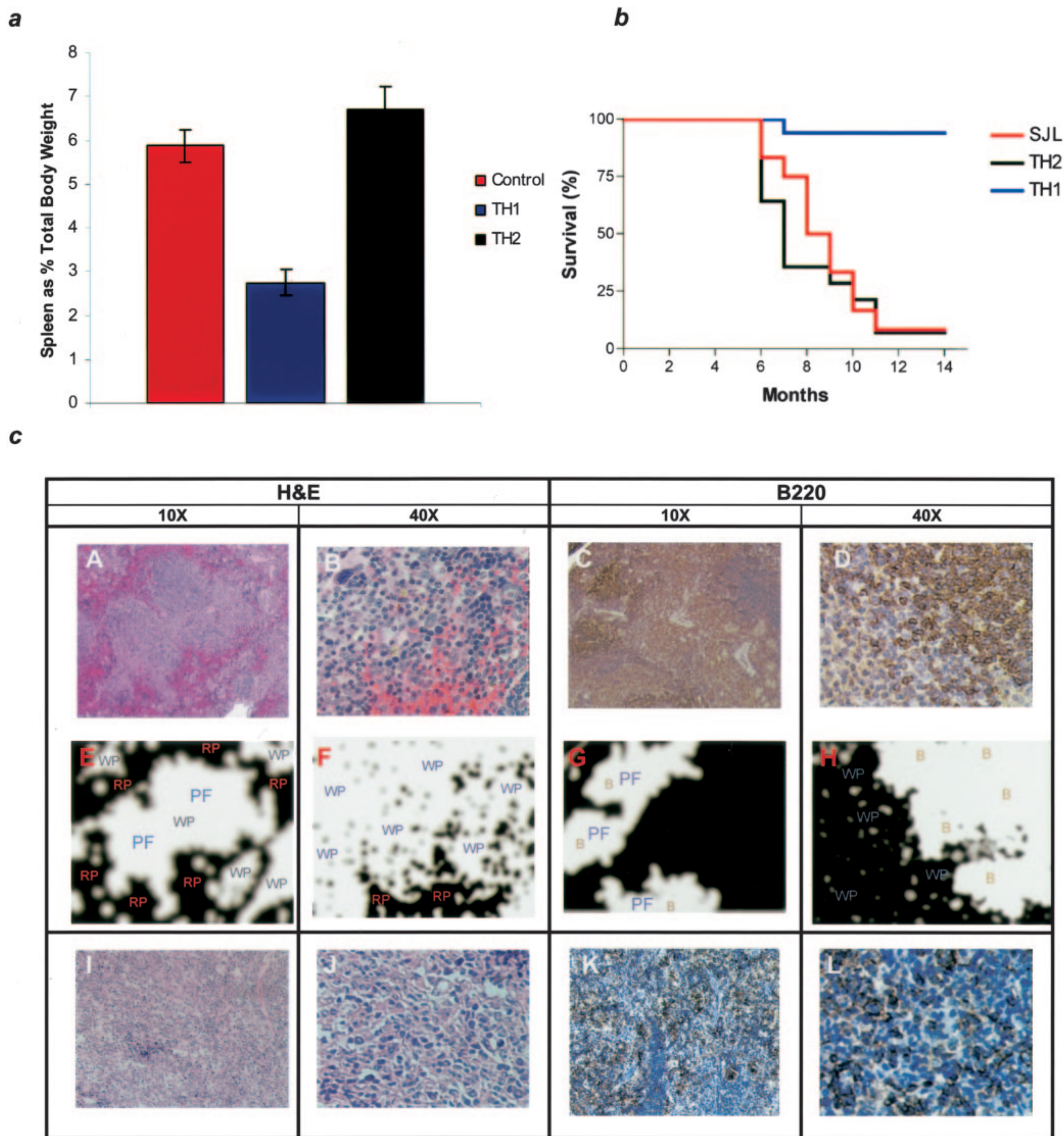

FIGURE 4. Th1 cells inhibit the development and growth of lymphoma cells in vivo. *a*, Th1 (A3.12; blue), but not Th2 (WB4; red), cells inhibit the in vivo growth of viable transplantable tumor. Eight-week-old mice were injected with 5×10^6 Th1 or Th2 cells. Ten days later, all mice were challenged with 2×10^4 purified (T cell-depleted) viable tumor cells. Tumor burden is presented as spleen weight as a percentage of total body weight. For comparison, spleen weight as a percentage of total body weight in nontumor-injected, age-matched control SJL mice is 0.61 ± 0.02 ($n = 5$). *b*, Th1 cells inhibit primary tumor development. Eight- to 12-wk-old mice were injected i.v. with $5–10 \times 10^6$ Th1 cells. The lymphoid organs of these mice were palpated twice monthly for a period of 14 mo. As the measure of survival shown in this figure, mice were killed when gross enlargement of secondary lymphoid organs reached the maximum size permitted by institutional guidelines. Tissues were then fixed in 10% formalin, and histological sections were analyzed for the presence of neoplastic cells. *c*, Aged SJL mice that received injections of Th1 cell lines at 12 wk of age (*a–d*) do not have neoplastic cells in their spleens and retain their splenic architecture in comparison with vehicle-injected control SJL mice (*i–l*). Tissues were fixed in 10% formalin and embedded in paraffin, and sections were stained with H&E (*a*, *b*, *i*, and *j*) or B220 HRP-conjugated mAb (*c*, *d*, *k*, and *l*). *e–h* were prepared directly from the micrographs shown in the *top row* of panels to indicate the prominent structural features in spleens from Th1-injected mice, including white pulp (WP), red pulp (RP), primary follicles (PF), and B cell areas (B).

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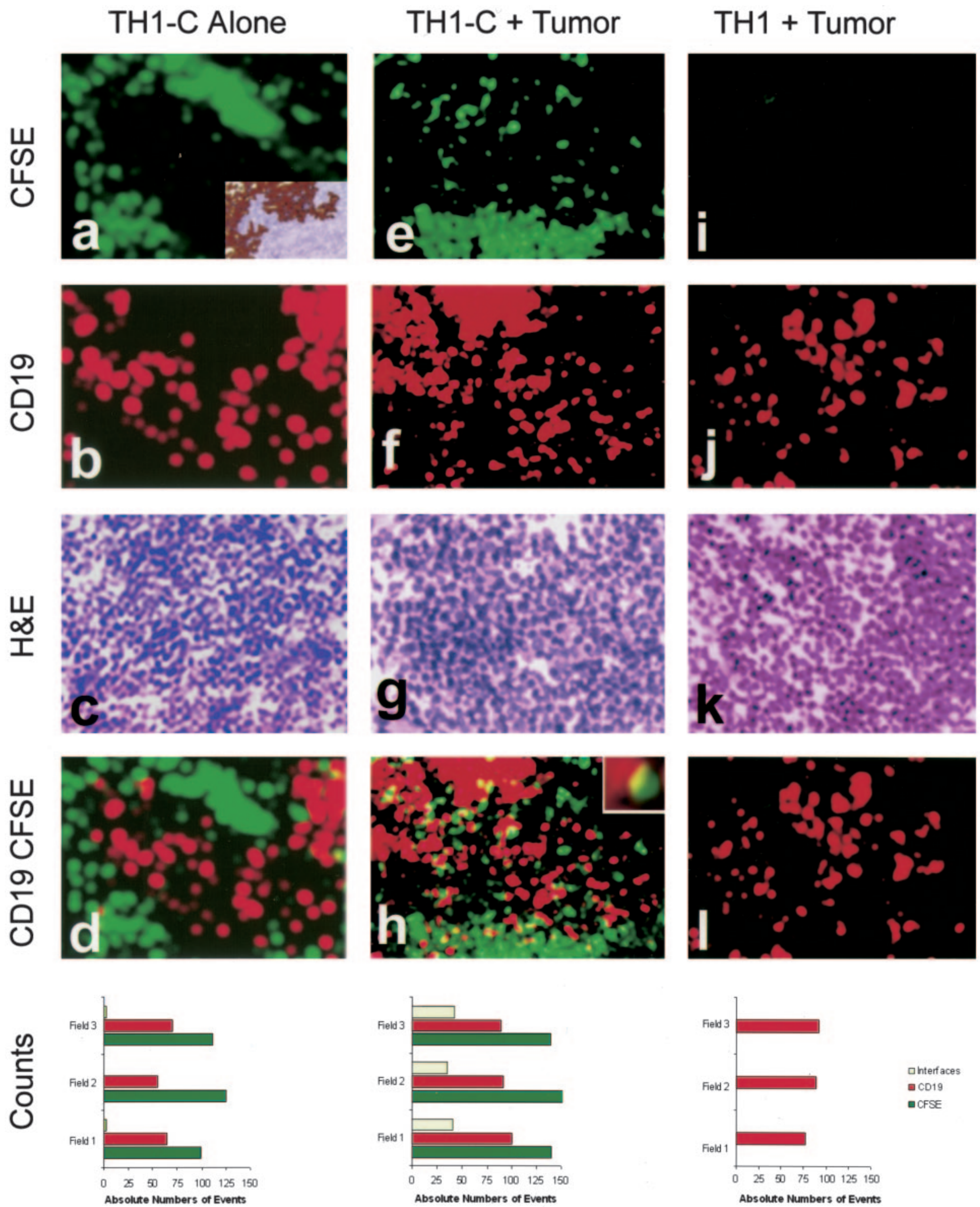

FIGURE 5. Migration and localization of Th cells into follicles are dependent upon the presence of tumor cells. In the absence of tumor (*a–d*), Th cells localize outside of the B cell areas of the spleen. Eight- to 12-wk-old SJL mice were injected i.v. with 5–10 × 10^6 CSFE-labeled Th1 (A3.12) cells; 90 days after adoptive transfer, mice were killed, and lymphoid tissues were snap-frozen and preserved at −80°C. CSFE-labeled Th1 cells were also $CD3^+$ (*inset*, *a*). Tissue sections were also stained with PE-conjugated (red) CD19 mAb (*b*, *f*, and *j*) Alternatively (*e–l*), some mice were challenged with 5 × 10^4 $CD19^+$ viable tumor cells for 48 h. Th1-tumor cell conjugates were examined by confocal microscopy, and interfaces (yellow bars and *inset*, *h*) of $CFSE^+$ Th1 cells and $CD19^+$ cells were quantitated (bar graphs). An enlarged merged image demonstrating an individual immunological synapse obtained from mice challenged with viable tumor is shown in *h*, *inset* (×100). Background fluorescence was determined by similar examination of spleen sections from mice in which unlabeled Th1 cells were injected (*i–l*). Representative single optical sections are shown in *a–l* at ×40 magnification.

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development of tumor-specific CTL. Studies to define regulatory cytokines other than IL-12 are currently under way.

Effective adoptive cellular immunotherapy requires Th cell survival and effector functions in vivo. Functional assays revealed that spleen cells obtained from mice that were previously injected for 1–2 wk with Th1 cells had the ability to develop cytotoxicity against tumor cells when restimulated ex vivo (Fig. 3*d*). In contrast, spleen cells from mice that had been injected for 3 or 4 wk with Th1 cells did not exhibit this ability. The Th1 cell lines that were injected for only 1 or 2 wk may have been sufficient in quantity to support the development of cytotoxicity upon in vitro stimulation, whereas after 3 and 4 wk in vivo in the absence of activation by tumor Ags, Th1 cells became quiescent and decreased in number. This speculation is supported by studies in which injected Th1 cells migrated to sites of inflammation and Ag, whereas nonactivated T cells immediately diminished in number and were found, albeit at low levels, in the spleen (39). Moreover, our data (Fig. 4*a*) clearly showed that mice injected with tumor-specific Th1, but not Th2, cells resisted tumor growth in vivo. Interestingly, others (40) suggest that Th2 cells may exhibit antitumor activity by attracting eosinophils, but the mechanism responsible for this cytotoxic activity was not identified. However, in our model, tumor-specific Th2 cells actually facilitated tumor growth (Fig. 4*a*). Therefore, our studies and others demonstrate an important role for Th1, but not Th2, cells in the development of tumor-specific cytotoxic effector cells (3, 41–43).

The in vivo model allowed us to address the influence of Th cells on tumor development. Mice injected with cells from Th1 lines showed a decreased incidence of primary tumors (Fig. 4*b*) and retained normal histological architecture in their secondary lymphoid tissues (Fig. 4*c*). It is unlikely, however, that introduction of Th1 cells prevented the transformation of normal cells to neoplastic cells. The results in Fig. 3*d* indicate that 3–4 wk after injection into young mice, the effector function of Th cells diminished, suggesting that tumor cells were required to maintain expanded numbers of CTLs. However, in aging mice it is likely that quiescent Th1 cells expanded as neoplastic cells developed and stimulated them. This possibility is supported by the data presented in Fig. 4, *b* and *c*, and Fig. 5, because the incidence of primary lymphomas in the recipients of Th1 cell lines was dramatically reduced.

Recent reports suggest the efficacy of tumor-reactive $CD8^+$ T cells (44) for adoptive immunotherapy. However, several complications have been noted, including lack of persistence (45), availability, expansion to high numbers, and immunosuppression-dependent persistence (44). Alterations in the signal pathways (46), including loss of $p56^{lck}$ and $p59^{fyn}$ activities as well as reduction in surface CD3γ and total loss of the CD3 ζ-chain, have also been reported. Our Th1 cell strategy, however, circumvented many of these complications, because host immunosuppression was not necessary to demonstrate anticancer activity (Fig. 4, *a* and *b*) and may, in fact, have inhibited cytotoxicity.

Tumor-specific Th1 cells have the capacity to migrate, infiltrate, and proliferate at sites of tumor. As a result, recruitment and activation of precursors into CTL effectors can proceed. In this context, Tim-3 would act as a surrogate marker to monitor the presence of Th1 cells, which would indicate the potential for the development of tumor-specific CTL and a favorable prognosis. Secondly, such a novel mechanism takes advantage of the entire repertoire of endogenous CTL precursors to mount a response to multiple tumor Ags. Therefore, in contrast to therapy with monospecific CTL lines, Th1 cellular therapy is more likely to result in tumor rejection due to the polyclonal quality of the ensuing endogenous CTL response. Indeed, resistance of tumors to conventional therapies due to mutation and expression of new tumor Ags has been described (47, 48). In this context, Th1, but not CTL, adoptive immunotherapy would continue to stimulate the production of tumor-specific CTL from the $CD8^+$ precursor repertoire against new tumor Ags. However, the survival of these lymphoma cells is dependent on the expression of the superantigen that stimulates Th2 cells to provide growth-promoting cytokines (27). Therefore, it is likely that even tumor cells that mutate the Ags recognized by CTL will continue to express the superantigen that stimulates Th1 cells that promote the generation of tumor-specific CTL.

Some human non-Hodgkin's B cell lymphomas arise from germinal center B cells, including follicular center cell lymphoma (small and large cell), diffuse large cell lymphoma, and Burkitt's lymphoma. These malignant B cells express HLA class II molecules, by which they can present Ags to infiltrating $CD4^+$ Th cells. Little is known about the interaction between these Th cells and tumor cells, the subsets of Th cells that respond (i.e., Th1 and/or Th2), and whether the outcome of the response favors tumor cell destruction or promotion of tumor cell growth. Because it is known that the formation of germinal centers is dependent on help from T cells (49, 50), it is possible that development and/or growth of human germinal center-derived B cell lymphomas will exhibit similar characteristics of T cell dependence. Indeed, TCR Vβ skewing of $CD4^+$ Th cells in tissues from patients with B cell lymphomas has been reported (51, 52).

Investigation of the $CD4^+$ Th cells that infiltrate selected types of human B cell lymphomas could yield insight into the role of the host immune system in the lymphomagenic process. However, given the impact of the engineered Th1 cells on the growth and development of effective CTL-mediated tumor immunity in our investigation, we anticipate that a translational study in humans would lead to the development of new and innovative strategies for cancer immunotherapy.

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