# CD8-Mediated Type 1 Antitumor Responses Selectively Modulate Endogenous Differentiated and Nondifferentiated T Cell Localization, Activation, and Function in Progressive Breast Cancer[1]

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**CD8 T cell-mediated immune responses fall into two distinct types based on effector cell-derived cytokine production. Type I CD8 T cells (Tc1) produce IFN-γ, whereas type 2 cells (Tc2) secrete IL-4, IL-5, IL-10, and GM-CSF. Using a murine TCR transgenic T cell/breast tumor model, we show that adoptively transferred Ag-specific Tc1 cells are more effective in delaying mammary tumor growth and progression than that of functionally distinct Tc2 cells. Donor Tc1 cells administered 7 days posttumor challenge localized and persisted at sites of primary tumor growth with antitumor responses that were dependent, in part, on effector cell-derived IFN-γ. Tc1-mediated responses markedly enhanced the appearance and local accumulation of highly differentiated ($CD44^{high}$) CD4 and CD8 endogenous tumor-infiltrating T cells when compared with that of untreated tumor-bearing mice. Conversely, Tc1 cell transfer markedly delayed the appearance of corresponding nondifferentiated ($CD44^{low}$) endogenous T cells. Such cells were acutely activated as defined by coexpression of surface markers associated with TCR engagement (CD69) and early T cell activation (CD25). Moreover, cellular response kinetics appeared to further correlate with the up-regulation of endogenous T cells producing the chemokine IFN-γ-inducible protein-10 in vivo. This suggested that CD8-mediated type 1 antitumor responses cannot only promote accumulation of distinct endogenous CD4 and CD8 T cell subpopulations, but also facilitate and preferentially modulate their localization kinetics, persistence, states of activation/differentiation, and function within the primary tumor environment at various stages of tumor progression. These studies offer insight into potential mechanisms for enhancing T cell-based immunotherapy in breast cancer.** ***The Journal of Immunology,*** **2006, 177: 8191–8201.**

Established breast cancer is a potentially dynamic and aggressive disease that is the second leading cause of cancer-related deaths among women in North America. Patients with advanced stage breast malignancies are not only less remedial to current therapies, but also possess a relatively high risk of relapse with time (1). This may be due in part to either the phenotypic and/or intrinsic properties of responding tumor-infiltrating T lymphocytes (TIL),[3] tumor-mediated evasion mechanisms that contribute to T cell dysfunction, and/or the presence/absence of pertinent cytokines and/or regulatory effector cells that may influence ensuing antitumor immune responses (2–13). Nonetheless, the phenotype, interaction, and immune function of the various T cell subpopulations at different stages of disease progression in patients with breast cancer remain relatively obscure.

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[3] Abbreviations used in this paper: TIL, tumor-infiltrating T lymphocyte; FAB, fluorescent Ab buffer; HA, hemagglutinin; IP-10, IFN-γ-inducible protein-10; Tc1, type I CD8 T cell; Tc2, type 2 CD8 T cell; WT, wild type.

CD8 T cells have been shown to play a major role in tumor eradication of many MHC class I-expressing tumors. Aside from their direct cytolytic potentials, differentiated CD8 effector T cells, which express up-regulated levels of CD44 and CD25, can be further classified into two distinct cell types based on their cytokine-secreting profiles following tumor Ag encounter (14–18). Type I CD8 T cells (Tc1) produce IFN-γ, whereas type 2 CD8 T cells (Tc2) secrete IL-4, IL-5, IL-10, and GM-CSF. Such cytokines cannot only have diverse inhibitory effects on tumor cells themselves, but also influence ensuing immune responses elicited by progressively growing malignancies. Although the existence of Tc1 and Tc2 cells has been demonstrated in patients with various clinical conditions (19–23), the nature and regulatory roles of these T cell subpopulations in tumor immunity and breast malignancies remain unclear. Previously, we have shown that these select CD8 T cell subpopulations and their functionally distinct antitumor responses can effectively regulate tumor growth and dissemination by multiple immune mechanisms (15, 24, 25). It is conceivable that because many cancers demonstrate considerable heterogeneity in the clinical course of their disease, treatment with tumor-reactive T cell-mediated immunotherapeutic agents, which potentially afford multiple and diverse mechanisms of tumor eradication, may enhance therapeutic benefits and provide a more favorable clinical outcome among patients with aggressive disease.

The application and characterization of cellular immunotherapeutic principles to the treatment of breast cancer is a relatively new undertaking (1, 6, 26, 27). In this study, we will investigate the nature and effectiveness of functionally distinct CD8-mediated Tc1 and Tc2 antitumor responses in progressive



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breast cancer and assess their effects on endogenous tumor-infiltrating T cell populations. We propose that adoptive T cell transfer with tumor-reactive Tc1 and/or Tc2 CD8 T cell subpopulations may not only be directly effective in treating established breast tumors, but also successful in modulating endogenous antitumor responses that induce tumor eradication and cure. Using a murine TCR transgenic T cell/breast tumor therapy model, we assessed the therapeutic effects and potential mechanisms of adoptively transferred tumor-reactive Tc1 and Tc2 CD8 T cell subpopulations in mice bearing established orthotopic breast tumors. Our results show that: 1) systemic transfer of Tc1 CD8 effector cells was more effective in delaying progressive tumor growth than that of corresponding treatment with Tc2 cells; 2) both donor Tc1 and Tc2 cells localized and progressively increased in cell number at local sites of primary tumor growth; 3) Tc1-, but not Tc2-mediated antitumor responses were dependent, in part, on effector cell-derived IFN-$\gamma$; and 4) donor Tc1 antitumor responses effectively promoted and selectively enhanced localization of both highly differentiated and nondifferentiated endogenous CD4 and CD8 TIL cell subpopulations to sites of established primary tumor growth. Furthermore, such endogenous T cell responses showed a capacity for enhanced TCR-mediated acute activation and heightened IFN-$\gamma$-inducible protein-10 (IP-10) chemokine gene expression within the tumor environment that was associated with delayed mammary tumor growth. We discuss the direct and indirect role(s) of tumor Ag-reactive Tc1-mediated responses in enhancing and/or modulating local endogenous T cell subpopulation infiltration kinetics, differentiation, and activation, and further provide a potential mechanism for IP-10-producing T cells during the effector phase of the type 1 antitumor response in progressive disease.

## Materials and Methods

### *Animals*

Female BALB/c mice (H-2$^d$), 6–10 wk of age, were obtained from The Jackson Laboratory. The clone 4 TCR transgenic mouse strain, on the BALB/c background (H-2$^d$), was originally obtained from L. Sherman (The Scripps Research Institute, La Jolla, CA). These mice express a transgenic TCR v$\beta$8.2/v$\alpha$10 specific for the IYSTVASSL peptide of hemagglutinin (HA) in the context of MHC class I, H2-K$^d$ (28). Homozygous IFN-$\gamma^{-/-}$ knockout mice, expressing the clone-4 TCR transgene (HA-BALB/c.IFN-$\gamma$ knockout), were generated by backcrossing HA-BALB/c mice onto syngeneic IFN-$\gamma$ knockout mice. Animals were maintained and treated according to animal care committee guidelines of the National Institutes of Health, Trudeau Institute, and Texas Tech University Health Science Center.

### *Tumor cells*

The TS/A tumor cell line, whose progression in vivo has been described previously as similar to human breast cancer, is an aggressive, poorly immunogenic murine mammary adenocarcinoma cell line of BALB/c origin that was originally generated by Nanni et al. (29). The parent TS/A tumor cell line expressing transfected HA (TS/A-HA) was generated using LipofectAMINE reagent (Invitrogen Life Technologies) following manufacturer's instructions. Briefly, the HA gene from the Mount Sinai strain of the PR8 influenza virus was subcloned into the $\beta$-actin expression vector obtained from B. Scott (Queen Elisabeth II Medical Center, Nedlands, Australia). Transfected cells were selected by culture in medium containing the neomycin analog geneticin (Invitrogen Life Technologies) at a final concentration of 500 $\mu$g/ml. The level of HA surface expression on transfected cells was measured by FACS analysis, using the biotinylated HA-specific mAb H36-4-5.2 that was provided by W. Gerhard (Wistar Institute, Philadelphia, PA). Clones expressing HA were sorted using a BD Biosciences FACSCalibur, and individual clones were generated by limiting dilution. Subconfluent monolayers of either parent TS/A or TS/A-HA, in log growth phase, were harvested with addition of 0.25% trypsin (Invitrogen Life Technologies) in HBSS and washed three times in DMEM containing 10% FCS (HyClone).

### *Generation of HA-specific CD8 effector T cell subpopulations*

To obtain CD8 effector T cells to HA peptide, single-cell suspensions from spleens and lymph nodes of HA-BALB/c mice were washed twice in HBSS and resuspended in DMEM 1640–10% FCS. CD8-enriched T cells were obtained by passing lymphoid cell suspensions through nylon wool columns and treating with anti-CD4 (RL172.4), anti-heat-stable Ag (J11D), anti-MHC class II (D3.137, M5114, CA4) mAbs, and complement. Small resting CD8 T cells were harvested from Percoll gradients (Sigma-Aldrich) and resuspended to appropriate cell concentrations in culture medium. Naive CD8 cells were typically >90% pure, as demonstrated by immunofluorescent Ab staining. APCs were enriched from spleens of normal BALB/c mice by anti-Thy 1.2 (HO13.14 and F7D5), anti-CD4 (RL172.4), and anti-CD8 (3.155) mAbs and complement. T cell-depleted APCs were pulsed with HA peptide (11 $\mu$M) for 30 min at 37°C and treated with mitomycin C (50 $\mu$g/ml; Sigma-Aldrich) for an additional 30 min at 37°C. For Tc1 effector cell generation, naive CD8 T cells from HA-BALB/c transgenic mice ($2 \times 10^5$ cells/ml) were stimulated with mitomycin C-treated HA peptide-pulsed APCs ($6 \times 10^5$ cells/ml) in the presence of IL-2 (20 U/ml; X63.IL-2 supernatants), IL-12 (2 ng/ml; provided by S. Wolf, Genetics Institute/Wyeth, Cambridge, MA), and anti-IL-4 mAb (200 U/ml; X63.Ag.IL-4 supernatants). Alternatively, for Tc2 effector cell generation, naive CD8 T cells from HA-BALB/c transgenic mice ($2 \times 10^5$ cells/ml) were stimulated with mitomycin C-treated HA peptide-pulsed APCs ($6 \times 10^5$ cells/ml) in the presence of IL-2 (20 U/ml), IL-4 (200 U/ml; X63.IL-4 supernatants), and anti-IFN-$\gamma$ mAb (20 $\mu$g/ml; XMG1.2). Effector T cell cultures were incubated for 4 days with additional IL-2 (20 U/ml) added to the cultures on day 2 to promote CD8 cell expansion of HA-specific Tc1 or Tc2 populations.

### *Adoptive T cell transfer model*

Syngeneic female BALB/c mice were injected s.c. with 0.2 ml of a single-cell suspension containing $1 \times 10^5$ TS/A-HA tumor cells in the right anterior mammary region. Seven days following tumor challenge, mice were treated with various doses of either transgenic TCR Tc1 or Tc2 HA Ag-specific effector T cells (i.v.), and tumor growth was monitored. Briefly, tumor growth was measured every 3 days using vernier calipers and volumes (mm$^3$) obtained by multiplying the measured length by the measured width by the calculated mean of these measured values. Data are presented as either the mean tumor volume ± SEM or as the tumor growth rate, which is the absolute value of the slope of the line as determined by regression analysis (95% confidence intervals). The ratio of the growth rates among groups of mice receiving different treatments to corresponding groups of untreated mice × 100 is depicted as the relative tumor growth rate. For detection of disease progression and metastases, cytospin preparations of single-cell suspensions from spleen, lung, and draining lymph nodes were obtained, fixed with methanol, and stained with eosin and methylene blue (Fisher Scientific). Although tumor cells appeared heterogeneous in size, they were easily differentiated as predominately larger cells with an elevated nuclear:cytoplasm ratio. Counts were performed on a total of 200–300 cells on coded slides.

### *Flow cytometric analysis*

Single-cell suspensions from TS/A-HA tumors were obtained by mechanical dispersion through nylon mesh cell strainers in DMEM-5% FCS and washed three times in fluorescent Ab buffer (FAB) consisting of 1% BSA and 0.02% sodium azide in 0.01 M PBS (pH 7.2). Immune cell populations were phenotyped by their expression of surface markers using either direct or indirect immunofluorescence staining techniques (18). Lymphocytes ($10^6$), pretreated with FcR block, were incubated for 20 min on ice with 100 $\mu$l of FAB containing 1 $\mu$g of various mAbs conjugated to either biotin, PE, FITC, or CyChrome. For biotinylated mAbs, streptavidin allophycocyanin or streptavidin CyChrome was used as a second-step reagent. The mAbs used include anti-CD90.1 (Thy 1.1; BD Pharmingen), anti-CD90.2 (Thy 1.2; BD Pharmingen), anti-CD8 (Caltag Laboratories), anti-CD4 (BD Pharmingen), anti-CD44 (BD Pharmingen; clone IM7), anti-CD3 (BD Pharmingen), anti-CD25 (BD Pharmingen), and anti-CD69 (BD Pharmingen). Stained cell preparations were then washed three times in FAB, and analyzed by multiparameter flow cytometry using a BD Biosciences FACSCalibur. For TS/A and TS/A-HA tumor cells, immunofluorescent staining took place with either FITC- or PE-conjugated anti-class I H2-K$^d$, anti-class I H2-D$^d$, anti-class II Ia$^d$, anti-HA (H36-4-5.2), anti-CD62P, or anti-CD44. Ten thousand cells were analyzed per sample with dead cells excluded on the basis of forward light scatter. Surface marker analysis was performed using CellQuest software, and the percentage of positive and absolute cell numbers was determined.

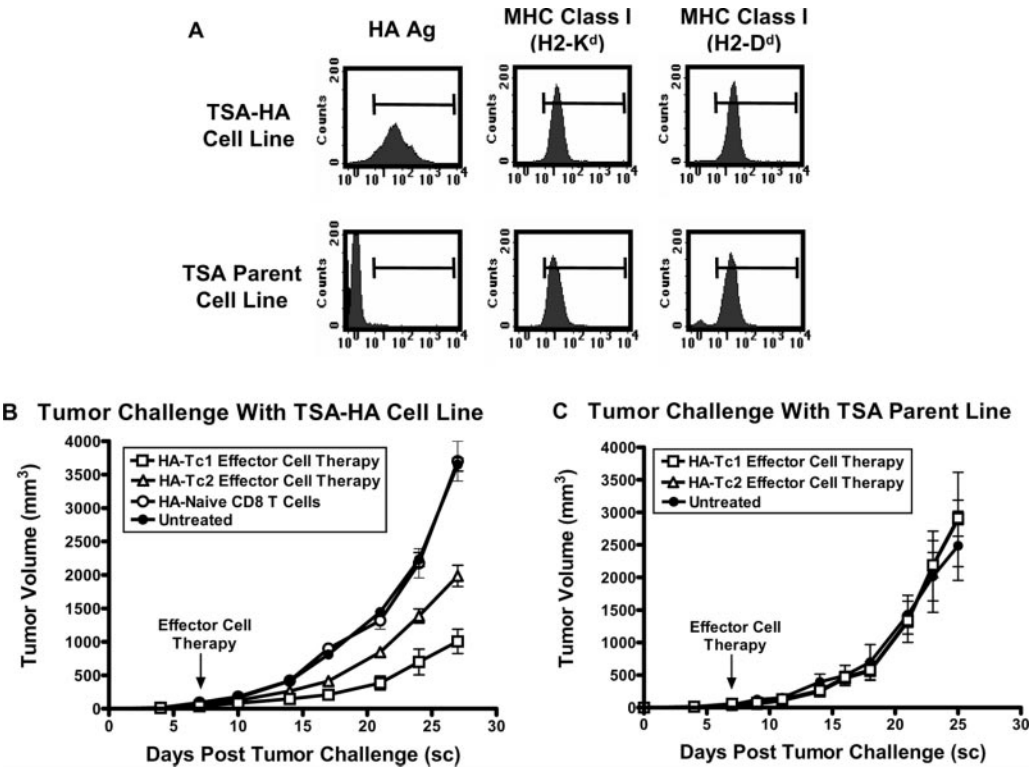


**FIGURE 1.** Characterization and therapeutic efficiency of adoptively transferred Ag-specific Tc1 and Tc2 effector cells in mice with established breast cancer. *A*, HA-transfected (TS/A-HA) and parent TS/A tumor cell lines were harvested in vitro and labeled with either anti-HA H36 mAb specific for HA Ag and FITC anti-rabbit IgG or MHC class I (H2-$K^d$ and H2-$D^d$). Live cells were distinguished by their forward/side light scatter profiles and analyzed by single-color flow cytometry. *B* and *C*, For in vivo studies, syngeneic mice ($n = 6–8$/group) were injected s.c. in the mammary fat-pad region with $1 \times 10^5$ TS/A-HA (*B*) or non-HA-expressing parent TS/A (*C*) tumor cells. Seven days later, HA TCR transgenic Tc1 or Tc2 effector cells ($1 \times 10^7$) were adoptively transferred (i.v.) into tumor-bearing mice, and tumor volumes were determined. Tumor volumes were measured using vernier calipers, and volumes were obtained by multiplying the measured length by the measured width by the calculated mean of these measured values. Results are representative of five independent experiments.

### *Analysis of endogenous T cell-derived chemokine mRNA expression in primary mammary tumor tissue*

Either freshly generated Tc1/Tc2 donor cells (Thy 1.2) or TIL cells from treated or untreated tumor-bearing recipient mice (Thy 1.1) were harvested, and single-cell suspensions from primary tumors were obtained at different time points following tumor challenge. T cells were enriched by negative selection using mAb and complement. Endogenous T cell suspensions were further treated with anti-Thy 1.2 mAb to eliminate donor cells and cultured with either nothing or plate-bound anti-CD3 for 5 h at 37°C. Total RNA from either unstimulated or plate-bound anti-CD3 cell cultures from Tc1 effector cell-treated or untreated tumor-bearing mice were prepared by tissue homogenation in TRIzol reagent (Invitrogen Life Technologies). mRNA levels were quantitated using the RiboQuant Multiprobe RNase protection assay system (BD Pharmingen) with the mCK-5 chemokine mRNA detection probe sets. Bands were detected using the Molecular Imager FX with the Quantity One Software analysis program (Bio-Rad) and normalized against the L32 housekeeping gene as relative units.

### *Statistical analysis*

For statistical analysis, two-tailed Student's *t* test, nonparametric Mann-Whitney *U* rank sum test, and/or Fisher's analysis were used when appropriate.

## Results

### *Characterization of the TS/A-HA tumor cell line*

The TS/A-HA tumor cell line was generated by transfecting the TS/A breast adenocarcinoma with influenza HA cDNA, as described in *Materials and Methods*. As shown in Fig. 1*A*, flow cytometric analysis showed that all TS/A-HA tumor cell lines expressed cell surface HA Ag, whereas parent TS/A cells showed no detectable levels. Moreover, both cell lines similarly expressed low, yet detectable, levels of surface MHC class I (H2-$K^d$ and H2-$D^d$) in vitro. Both parent and TS/A-HA tumor cells progressively grew in vivo without evidence of spontaneous regression when injected s.c. into mammary fat-pads of normal syngeneic BALB/c mice. Concomitantly, regional lymph node and systemic tumor involvement were determined by either gross or microscopic assessment. Metastases was initially present in draining lymph nodes by days 17–21 following tumor challenge and progressively disseminated to lungs with time. Collectively, these studies establish and characterize, in part, a select Ag-expressing orthotopic tumor model that correlates with both local-regional (early) and/or systemic (late) stages of progressive breast cancer.

### *Phenotypic characterization of cytokine-polarized HA-specific Tc1 and Tc2 CD8 T cell subpopulations*

CD8 Tc1 and Tc2 effector T cells were generated ex vivo from HA-specific TCR transgenic HA-BALB/c mice, as described in *Materials and Methods*. As shown in our earlier studies (18, 30), both T cell subpopulations demonstrated potent tumor Ag-specific cytolytic activity to HA peptide-expressing tumor cell targets that was predominately mediated through the perforin pathway in vitro. Moreover, Tc2 effector cell subpopulations released considerable amounts of IL-4, IL-5, IL-10, and GM-CSF upon restimulation with HA peptide-pulsed APC or tumor. In contrast, Tc1 effector

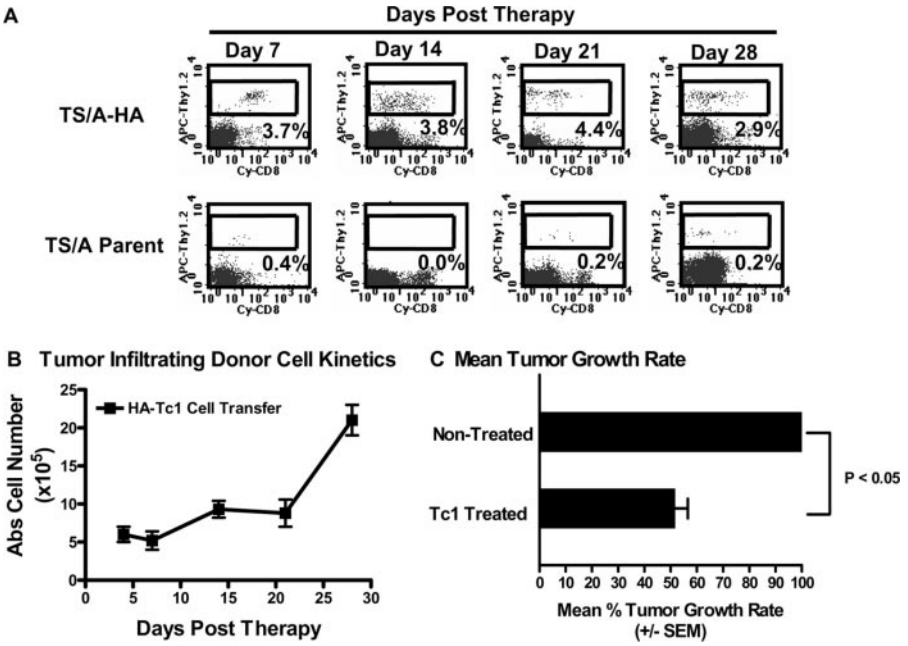


**FIGURE 2.** Accumulation and localization kinetics of donor tumor-reactive Tc1 effector cells in mice with established mammary tumors. TS/A-HA- or non-HA-expressing TS/A tumor-bearing mice (Thy 1.1) were treated with $1 \times 10^7$ Tc1 effector cells (Thy 1.2), as described in *Materials and Methods*. At specified time points following Tc1 cell transfer, tumors from groups of mice ($n$ = 2–3 mice/group/time point) were harvested, and single-cell suspensions were labeled with anti-Thy 1.2 and CD8 mAbs (*A*). Numbers represent the percentages of tumor-infiltrating donor cell populations as determined by multiparameter flow cytometry. *B*, Absolute cell numbers of donor Tc1 cells were calculated as the percentage of positively stained Thy 1.2/CD8 T cells × the total number of monocytes/tissue. Data are expressed as the mean ± the SEM of two to three mice per time point in three independent experiments. *C*, Tumor volumes were measured using vernier calipers, and the relative tumor growth rates among treated and nontreated groups of mice were determined by regression analysis (95% confidence intervals), as described in *Materials and Methods*. Results are expressed as the mean ± the SEM of three independent experiments.

cells produced substantial amounts of IFN-γ with no detectable levels of Tc2-like cytokines. Flow cytometric analysis showed that both effector cell populations expressed similar patterns of cell surface Ag markers that are characteristic of effector cell phenotype (31). Effector cell populations were $CD8^+CD4^-$, Thy $1.2^+$ and expressed up-regulated levels of both CD44 and CD25 and down-regulated levels of CD62L.

### *Cytokine-polarized Ag-specific Tc1 or Tc2 effector cell subpopulations delay mammary tumor growth and progression in mice with established breast malignancy*

To initially address the potential therapeutic role of functionally distinct HA tumor Ag-specific Tc1 and Tc2 effector T cell subpopulations in established breast malignancies, we generated and used a TCR transgenic T cell/breast tumor therapy model. Following TS/A-HA tumor challenge, ex vivo generated CD8 effector T cell subpopulations were transferred i.v., and therapeutic efficacy was evaluated by tumor volume and growth rate analysis, as described in *Materials and Methods*. As shown in Fig. 1*B*, both Tc1 and, to a lesser extent, Tc2 effector cell subpopulations significantly ($p < 0.05$) delayed TS/A-HA mammary tumor cell growth in mice with 7-day established orthotopic tumors. Moreover, transfer of similar numbers of naive pre-effector CD8 T cells ($CD44^{low}$/$CD62L^{high}$/$CD69^-$/$CD25^-$) from HA-BALB/c mice had no effect on tumor reduction and/or growth among mice bearing established mammary tumors. Concomitantly, we assessed the immunological specificity of HA-Ag-specific Tc1 and Tc2 effector cell therapy in vivo. As shown in Fig. 1*C*, transfer of Tc1 and Tc2 effector cells into mice challenged with the non-HA-expressing TS/A parent line showed no detectable therapeutic effect on tumor growth when compared with that of untreated control TS/A tumor-bearing mice. Although both effector cell therapies appeared relatively effective and highly Ag specific, they were nonetheless ineffective in establishing total long-term tumor eradication as breast tumor growth and progression among treated animals did not abate (Fig. 1). These studies suggest that phenotypically distinct tumor-reactive Tc1 or Tc2 effector cell subpopulations did effectively and specifically participate in T cell-mediated antitumor responses, however, with both different efficiencies and with limited success in the treatment of progressive breast cancer. Similar results were obtained in four other independent experiments.

### *Localization of adoptively transferred Ag-specific Tc1 effector cells to sites of tumor growth is tumor Ag specific*

Because therapeutic efficacy by adoptive T cell transfer is largely dependent on the localization and persistence of donor cells at the site of tumor growth, we next attempted to assess the local accumulation and kinetics of donor Tc1 cell subpopulations in primary tumors. Using Thy 1.1 congenic mice to distinguish between donor (Thy 1.2) and recipient (Thy 1.1) CD8 T cell populations, mammary tumors were harvested from mice at various time intervals following Tc1 effector cell treatment, and donor cells were enumerated by multiparameter flow cytometric analysis. As shown in Fig. 2, donor Ag-specific Tc1 effector cells (Thy 1.2/CD8) accumulated at the tumor site among mice with established mammary tumors as early as day 7 with peak proportion levels at day 21 following treatment. Moreover, their cell numbers increased and correlated with a significant decrease in the rate of tumor growth among treated animals when compared with that of nontreated

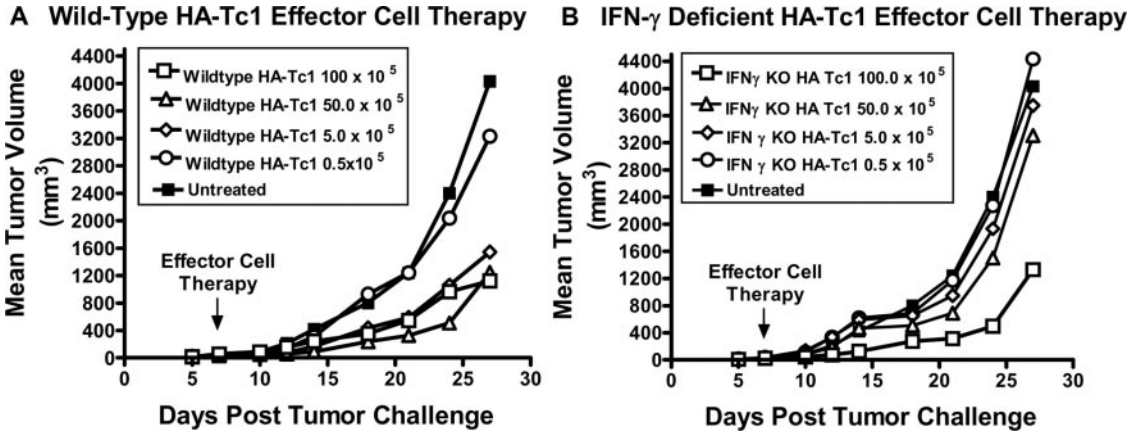


**FIGURE 3.** Therapeutic role of Tc1 effector cell-derived IFN-γ in mice with established mammary tumors. Syngeneic mice ($n = 6–8$/glycoprotein) were injected s.c. in the mammary fat-pad region with $1 \times 10^5$ TS/A-HA tumor cells. Seven days later, various doses of WT (*A*, *inset*) or IFN-γ-deficient (*B*, *inset*) HA-specific Tc1 effector cells were adoptively transferred (i.v.) into tumor-bearing mice, and tumor volumes were determined as described in Fig. 1. Data are representative of two independent experiments with similar results.

tumor-bearing control groups (Fig. 2, *B* and *C*). In contrast, negligible proportions (<0.5%) of donor Tc1 cells were observed in corresponding groups of mice receiving non-HA-expressing TS/A parent tumor cells. Similar results were observed in Tc2-treated animals (data not shown). Interestingly, we observed a substantial down-regulation in CD8 Ag expression among both donor Tc1 and Tc2 TIL cells with time. Although we are not in a position to definitively address this, we speculate that this may reflect that transferred cells become functionally impaired, in part, and provide a rationale for the observation that therapeutic efficacy by adoptively transferred T cells is limited. Nonetheless, our data suggested that both effector cell subpopulations did accumulate and localize at sites of primary tumor growth and that such trafficking was highly Ag specific.

### *Role of donor effector cell-derived IFN-γ in Tc1-mediated breast tumor therapy*

Because Tc1 cells produced substantial amounts of IFN-γ and persisted within the tumor environment with time, we quantitatively analyzed the role and antitumor effects of effector cell-derived IFN-γ. Briefly, different effector cell concentrations of Tc1 cells derived from either wild-type (WT) or IFN-γ-knockout HA-TCR transgenic BALB/c mice were used to treat syngeneic mice with established 7-day orthotopic TS/A-HA mammary tumors. As shown in Fig. 3, mice receiving doses of $50–100 \times 10^5$ of WT HA-specific effector cells showed a decrease in tumor growth and progression when compared with that of untreated tumor-bearing control animals. Moreover, transfer of 10-fold less WT Tc1 effector cells, at numbers as low as $5 \times 10^5$, resulted in a similar therapeutic effect. In contrast, groups of mice receiving a similar dose of IFN-γ-deficient Tc1 cells showed no therapeutic effect, and only when given a 20-fold higher effector cell number ($100 \times 10^5$) did animals start to show substantial decreases in breast tumor cell growth (Fig. 3*B*). In contrast, mammary tumor growth among mice treated with IFN-γ-deficient Tc2 effector cell subpopulations was not different from that of corresponding groups of tumor-bearing mice receiving WT Tc2 effector cell transfer (data not shown). These results suggest that on a per cell basis, donor cell-derived IFN-γ plays a substantial role in Tc1, but not Tc2, effector cell-mediated antitumor responses in animals with progressive breast malignancy.

### *Donor Tc1 effector cells influence the accumulation, localization kinetics, and ratios of differentiated and nondifferentiated endogenous CD8 and CD4 TIL cell subpopulations during progressive mammary tumor growth*

Because it has been shown previously that adoptively transferred CD8 effector T cells can potentially influence immune cell infiltration and function within the tumor environment, we next assessed and characterized the local (tumor) and systemic (spleen) distribution of differentiated and nondifferentiated endogenous T cell subpopulations following Tc1 effector cell transfer. Congenic BALB/c/Thy 1.1 recipient mice were injected s.c. in the mammary region with $1 \times 10^5$ HA-expressing TS/A breast adenocarcinoma tumor cells. Seven days later, when mammary tumors were established, $1 \times 10^7$ HA Ag-specific Tc1 effector cells, generated from Thy 1.2 HA-BALB/c mice, were systemically transferred into tumor-bearing mice, and endogenous Thy 1.1 TIL cell subpopulations coexpressing various levels of CD44 were enumerated by multiparameter flow cytometry. Because up-regulated CD44 expression is indicative of T cell differentiation following Ag encounter (31), we assessed the numbers and frequencies of recipient Thy 1.1/$CD8^+$ T cell subpopulations coexpressing elevated levels of CD44 surface Ag (Thy 1.1/$CD8^+$/$CD44^{high}$) at various stages of progressive mammary tumor growth. As shown in Fig. 4, *A* and *E*, the absolute cell numbers and frequencies of endogenous Thy 1.1/$CD8^+$/$CD44^{high}$ cells in primary tumors of mice receiving Tc1 effector cell therapy progressively increased with significantly ($p < 0.05$) greater levels at later time points (days 20–30 post-tumor challenge) following effector cell transfer when compared with that of corresponding groups of untreated tumor-bearing control mice. In parallel studies, corresponding endogenous $CD4^+$ TIL cell (Thy 1.1/$CD4^+$/$CD44^{high}$) numbers were not only significantly ($p < 0.05$) greater than that in groups of untreated control mice, but also markedly greater than that of corresponding endogenous CD8 TIL cells from both treated and nontreated animals (Fig. 4, *A* and *B*). However, T cell frequencies among endogenous CD4/$CD44^{high}$ lymphocytes were greater at early time points following T cell transfer and decreased with time (Fig. 4*F*), whereas CD8/$CD44^{high}$ frequencies remained similar at corresponding time points (Fig. 4*E*). In contrast, spleens from either treated or nontreated animals showed no significant ($p > 0.05$) differences in either endogenous CD4 or CD8 T cell numbers or frequencies

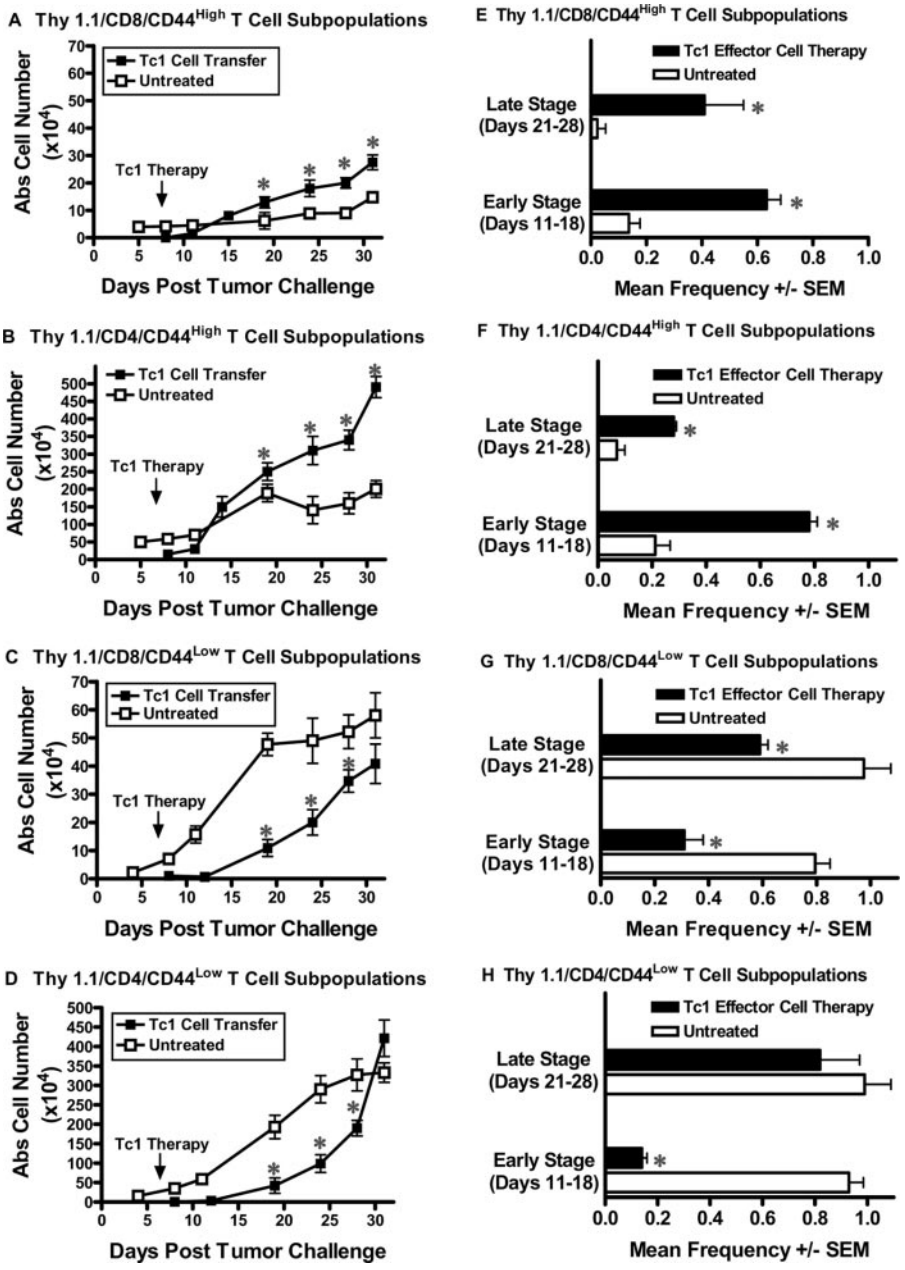


**FIGURE 4.** Cell number and frequency kinetics of differentiated and nondifferentiated endogenous CD8 and CD4 TIL cell subpopulations following Ag-specific Tc1 effector cell transfer. Recipient TS/A-HA tumor-bearing mice (Thy 1.1) were treated 7 days following tumor challenge with $1 \times 10^7$ Tc1 effector cells (Thy 1.2), as described in Fig. 2. Tumors were harvested at specified time points from either untreated or effector cell-treated groups of mice, and single-cell suspensions were obtained and labeled with anti-Thy 1.1, anti-CD4, or anti-CD8 and anti-CD44 mAbs. Gates were set on Thy $1.1^+$ and $CD4^+$ or $CD8^+$ T cell populations, and $CD44^{high}$ (differentiated) and $CD44^{low}$ (nondifferentiated) staining profiles within these populations were assessed by multiparameter flow cytometry. The absolute cell numbers of differentiated (*A* and *B*) and nondifferentiated (*C* and *D*) endogenous Thy $1.1^+$/$CD4^+$ or Thy $1.1^+$/$CD8^+$ TIL cells are shown. Results are expressed as the mean ± SEM of three to four mice per group/time point in three independent experiments. *E–H*, The frequencies of corresponding CD8 and CD4 T cell subpopulations at early (days 11–18) and late (days 21–28) stages following tumor challenge among treated and nontreated mice. The mean frequency is defined as the absolute cell numbers of either Thy $1.1^+$/$CD4^+$ or Thy $1.1^+$/$CD8^+$ TIL cells coexpressing high or low levels of CD44 surface Ag per the absolute cell numbers of total Thy 1.1/$CD4^+$ or Thy 1.1/$CD8^+$ T cells, respectively. Results are expressed as the mean ± the SEM of specified cell frequency values of three to four mice per group per designated time point in three independent experiments. ∗, Value of $p$ for treated vs untreated animals at corresponding time points: $p < 0.05$.

(data not shown). This suggested that Tc1 cell transfer can locally facilitate and enhance both differentiated endogenous CD4 and CD8 T cell subpopulation numbers and modulate localization and frequency kinetics within the primary tumor environment during progressive tumor growth.

In contrast, the frequency and absolute cell numbers of endogenous nondifferentiated Thy 1.1/CD8/$CD44^{low}$ TIL cells among untreated tumor-bearing animals were greater when compared with that of differentiated endogenous Thy 1.1/CD8/$CD44^{high}$ TIL cell subpopulations in these same animals (Fig. 4, *A* and *E* vs *C* and *G*, respectively). However, nondifferentiated CD8 TIL cell frequencies and cell numbers were significantly lower following donor Tc1 cell transfer and showed, for the most part, a substantial delay in their localization kinetics when compared with that of untreated tumor-bearing control mice (Fig. 4, *C* and *G*). In parallel studies, nondifferentiated CD4 T cell numbers and frequencies showed similar results with lower cell numbers in Tc1-treated mice at both early and late stages of tumor progression (Fig. 4, *D* and *H*). Moreover, the mean absolute cell number ratios of nondifferentiated (CD4/$CD44^{low}$) to differentiated (CD4/$CD44^{high}$) endogenous TIL cell subpopulations among groups of Tc1-treated tumor-bearing mice (0.47 ± 0.14) at later stages of tumor progression (>day 20) were significantly ($p < 0.005$) lower and nearly 4-fold less than that of corresponding nontreated (1.71 ± 0.23) mice. Similarly, corresponding ratios of endogenous CD8 TIL cell subpopulations were significantly lowered in these same animals following Tc1 effector cell transfer (1.28 ± 0.18 vs 5.73 ± 0.77 for treated and untreated groups of mice, respectively; $p < 0.001$). This suggested that: 1) mammary tumors from untreated animals induced an early and prolonged accumulation of nondifferentiated CD4 and CD8 T cell subpopulations; 2) adoptively transferred Tc1 effector cell-mediated antitumor responses can influence and/or modulate the frequency and localization kinetics of less differentiated T cell subpopulations during progressive tumor growth; and 3) donor Tc1 effector cells qualitatively and/or quantitatively enhanced the accumulation, proportion, and localization kinetics of

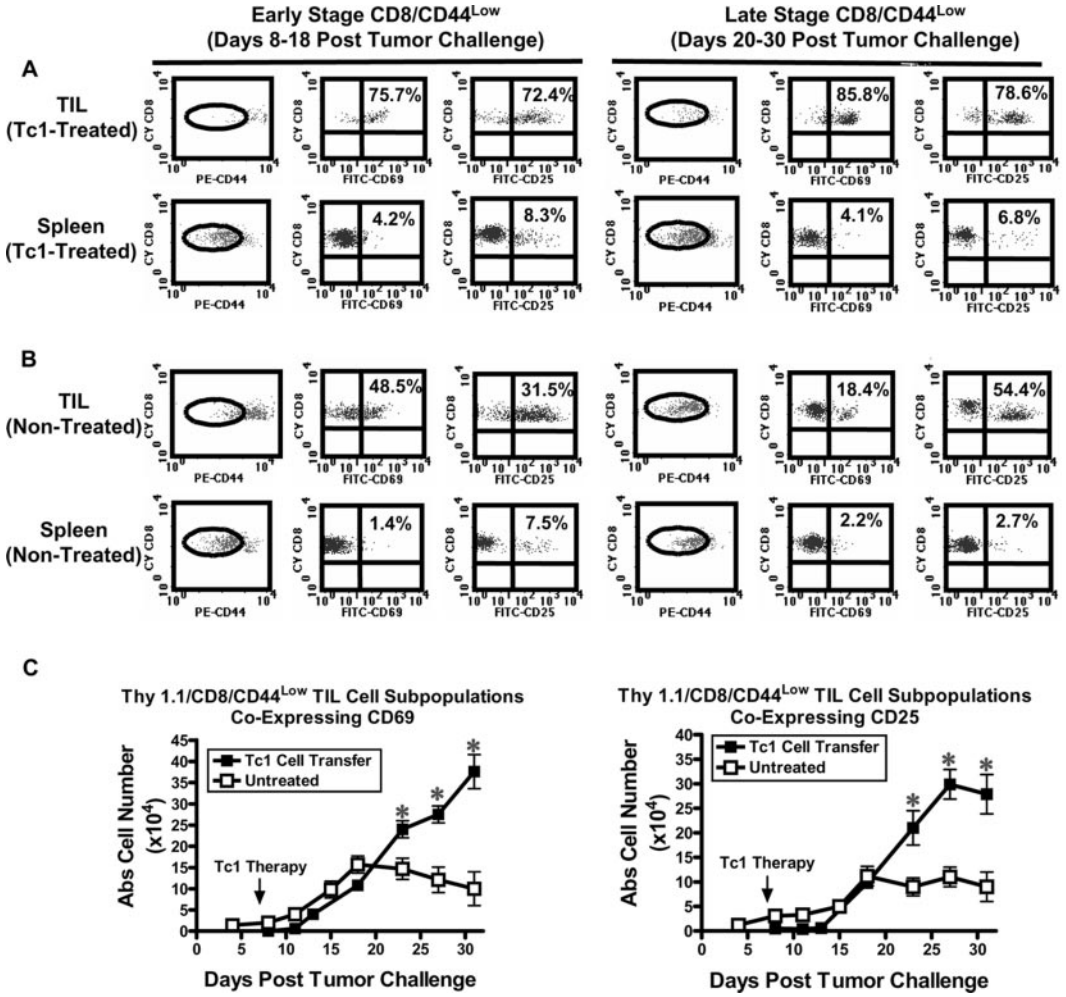


**FIGURE 5.** Tc1 effector cell transfer promotes and enhances levels of acutely activated endogenous $CD8^+/CD44^{low}$ TIL cell subpopulations that correlate with delays in late-stage mammary tumor cell growth in vivo. *A* and *B*, Tumor-bearing mice (Thy 1.1) were treated with Tc1 effector cells (Thy 1.2), and both tumors and spleens were harvested from effector cell-treated (*A*) or nontreated (*B*) groups of mice at either early (days 8–18) or late (days 20–30) time points following tumor challenge. Single-cell suspensions were labeled with anti-Thy 1.1, anti-CD8, anti-CD44, and anti-CD69 or anti-CD25 mAbs. Gates were set on Thy $1.1^+/CD8^+/CD44^{low}$ TIL cell subpopulations, and surface marker profiles, associated with TCR engagement (CD69) and/or early acute activation (CD25), within these populations were assessed by multiparameter flow cytometry. Numbers represent the percentages of recipient-derived Thy $1.1^+/CD8^+/CD44^{low}$ T cell populations coexpressing CD69 and CD25 surface Ag at respective time points following Tc1 cell transfer. *C*, The absolute cell numbers of acutely activated T cell subpopulations within these same animals at specified time points following therapy were assessed, as described in Fig. 2. Results are expressed as the mean ± SEM of three to five mice per time point for each group in three independent experiments. *, Value of $p$ for treated animals vs corresponding untreated animals: $p < 0.01$.

highly differentiated subpopulations of both endogenous CD4 and CD8 cells within the primary tumor environment over time.

### *Endogenous nondifferentiated CD4/CD44$^{low}$ and CD8/CD44$^{low}$ TIL cell subpopulations accumulate and undergo acute activation at sites of primary tumor growth following Ag-specific Tc1 cell transfer*

Sites of local inflammation have been associated, in part, with various stromal and/or homeostatic regulatory components that can profoundly modify immune cell recruitment, phenotype, and function, and thus result in the accumulation of ineffective naive and/or functionally erroneous T cell subpopulations at such sites (32–37). Because we have identified a substantial influx and progressive elevation in endogenous nondifferentiated $CD44^{low}$ T cell subpopulations at sites of primary tumor growth, we next investigated whether such cells can respond and/or undergo acute activation following Tc1 effector cell transfer. To address this, we evaluated such endogenous TIL cell subpopulations for surface markers associated with acute TCR engagement (CD69) and/or early stage T cell activation (CD25) in vivo (31, 36, 38–40). As shown in Fig. 5, the frequency and proportions of endogenous $CD8/CD44^{low}$ TIL cells coexpressing CD69 and CD25 were consistently elevated at all time intervals tested, with the most notable effects occurring at later stages following effector cell transfer when compared with that of corresponding cell populations in untreated tumor-bearing control animals. Because we found low, yet significant, donor cell numbers in the spleens of these animals, we investigated the effects of i.v. transferred Tc1 cells on systemic T cell populations distal to tumor growth. The frequency and proportion of corresponding cell subpopulations in spleens of these same mice were consistently lower than those at sites of local primary tumor growth. Similar results were obtained with corresponding endogenous $CD4/CD44^{low}$ T cell subpopulations (Fig. 6). This suggested that endogenous nondifferentiated CD4 and CD8 TIL cell subpopulations could effectively recognize

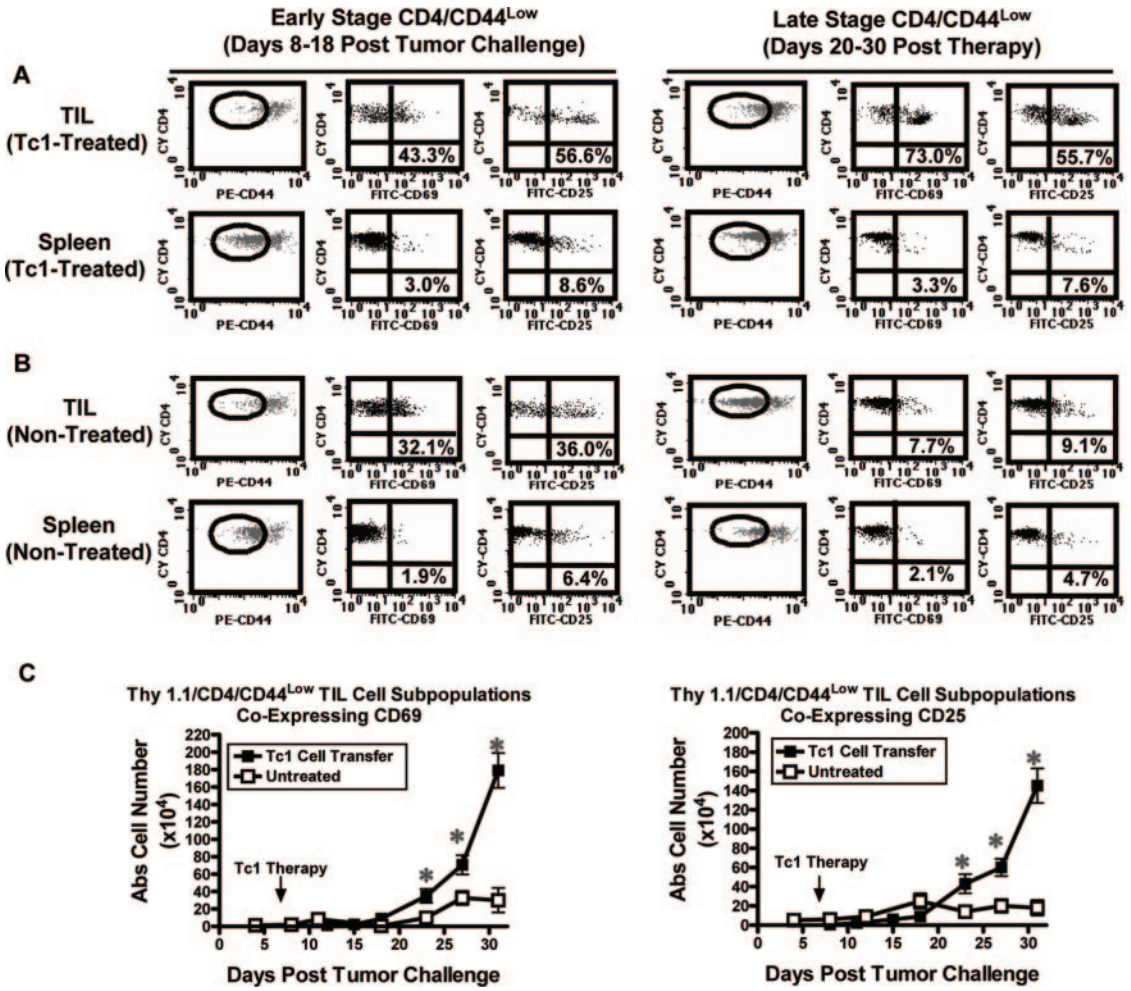


**FIGURE 6.** Tc1 effector cell transfer promotes and enhances levels of acutely activated endogenous $CD4^+/CD44^{low}$ TIL cell subpopulations that correlate with delays in late-stage mammary tumor cell growth in vivo. *A* and *B*, Tumor-bearing mice (Thy 1.1) were treated with Tc1 effector cells (Thy 1.2), and both tumors and spleens were harvested from either effector cell-treated (*A*) or untreated (*B*) groups of mice at either early (days 8–18) or late (days 20–30) time points following tumor challenge. Single-cell suspensions were obtained and labeled with anti-Thy 1.1, anti-CD4, anti-CD44, and anti-CD69 or anti-CD25 mAbs. Gates were set on Thy $1.1^+/CD4^+/CD44^{low}$ TIL cell subpopulations, and CD69- and CD25-staining profiles within these populations were assessed by flow cytometry. Numbers represent the frequency of acutely activated Thy $1.1^+/CD4^+/CD44^{low}$ T cell subpopulations coexpressing CD69 and CD25 surface Ags at specified time points. *C*, The absolute cell numbers of acutely responding T cell subpopulations within these same animals at specified time points following therapy were assessed, as described in Fig. 5. Results are expressed as the mean ± SEM of three to five mice per time point for each group in three independent experiments. ∗, Value of $p$ for treated animals vs corresponding untreated animals: $p < 0.01$.

and locally respond to TCR-Ag-induced activation at sites of primary tumor growth following Tc1 effector cell transfer. As expected, nearly all (>88%) differentiated $CD8/CD44^{high}$ and $CD4/CD44^{high}$ T cell subpopulations expressed CD69 and CD25 activation markers at both early and late stages of progressive tumor growth in both untreated and Tc1-treated mice (data not shown). Collectively, this suggested that Tc1 cell transfer can effectively facilitate and/or enhance the accumulation of acutely activated CD4 and CD8 T cell subpopulations that potentially affect localized antitumor responses during primary tumor cell growth and maturation.

*Adoptively transferred tumor-reactive Tc1 effector cells enhance endogenous T cell-derived IP-10 expression within the tumor environment that correlates with both endogenous T cell localization and delayed mammary tumor growth*

IP-10 has been shown to be expressed by T cells that aid in enhancing local type 1-like T cell-mediated immune responses at sites of inflammation (41–47). Because we have shown that adoptively transferred Ag-specific Tc1-mediated antitumor responses were notably dependent on IFN-γ and profoundly facilitated the local accumulation of phenotypically distinct endogenous T cell subpopulations within the mammary tumor environment, we next assessed the expression kinetics of endogenous T cell-derived IP-10 following Tc1 cell transfer. Endogenous TIL cells (Thy 1.1) were enriched and treated ex vivo with anti-Thy 1.2 mAb and complement to eliminate donor T cells from either untreated or Tc1-treated tumor-bearing mice. Recovered cells were restimulated with plate-bound anti-CD3 mAb, and IP-10 chemokine gene expression was assessed by RNase protection assays. As shown in Fig. 7, groups of untreated TS/A-HA tumor-bearing mice showed an early (4–10 days posttumor challenge) elevation in IP-10 gene expression that precipitously declined with tumor progression. In contrast, Tc1-treated tumor-bearing mice showed a similarly early and more prolonged elevation (1–8 days post-Tc1 cell transfer) of such chemokine in vivo. Moreover, a secondary biphasic IP-10 chemokine response occurred within groups of these same mice

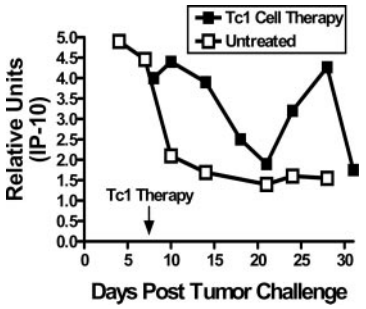


**FIGURE 7.** Endogenous T cell-derived IP-10 chemokine gene expression profiles in mammary tumors following Tc1 effector cell therapy. Mammary tumors from either Tc1-treated or untreated tumor-bearing mice ($n$ = 2/time point/experiment) were harvested, and single-cell suspensions were obtained, as described in Fig. 2. Endogenous T cells were further enriched by treatment with anti-Thy 1.2 and complement in vitro to eliminate adoptively transferred donor cells. Resulting T cell populations were cultured with either nothing or plate-bound anti-CD3 for 5 h at 37°C. T cell cultures were harvested and total RNA was prepared, as described in *Materials and Methods*. IP-10 chemokine mRNA was detected by RNase protection assays and normalized against the L32 housekeeping gene as relative units for comparative analysis. Data are representative of two independent experiments with similar results.

at later time points (14–21 days) following Tc1 cell transfer. Because Thy 1.2 donor cells were depleted and freshly generated Tc1 effector cells showed no detectable levels of IP-10 expression following restimulation with anti-CD3 in vitro (data not shown), we suggest that adoptively transferred Tc1 effector cells effectively promoted and facilitated the accumulation of endogenous T cells expressing up-regulated levels of IP-10.

## Discussion

In this study, we developed and characterized a murine HA-Ag breast tumor model to evaluate tumor-reactive Tc1 and Tc2 effector cell-mediated antitumor responses and their effects on endogenous T cell subpopulations in progressive breast cancer. Using HA Ag-specific TCR transgenic mice, we generated and adoptively transferred Tc1 and Tc2 CD8 effector cells into syngeneic tumor-bearing mice and relate that: 1) substantial numbers of systemically transferred Tc1 or Tc2 effector cell subpopulations preferentially accumulated at the site of mammary tumor growth; 2) single dose treatment with tumor-reactive Tc1 cells was more effective in delaying mammary tumor growth and progression than that of functionally distinct Tc2 cells; and 3) antitumor responses by Tc1, but not Tc2, effector cells were highly dependent on donor cell-derived IFN-$\gamma$. Although this is in agreement with previous studies performed by us and others using other tumor models (18, 24, 48–53), we next extended our observations to quantitatively and qualitatively assess endogenous CD4 and CD8 TIL cell subpopulation activation, differentiation, localization kinetics, and potential antitumor mechanisms at various stages of breast tumor progression following CD8 effector cell transfer. We show that Ag-specific CD8-mediated type 1 antitumor responses selectively facilitate the localization and activation potentials of differentiated ($CD44^{high}$) and nondifferentiated ($CD44^{low}$) endogenous CD4 and CD8 T cell subpopulations at various stages of tumor progression. Such cellular response kinetics appeared to further correlate with the up-regulation of endogenous T cells producing the chemokine IP-10 in vivo. This suggested that Tc1-mediated antitumor responses may be dependent on several direct and indirect mechanisms that initiate and orchestrate select endogenous T cell responses that influence the effector phase of the immune response to breast cancer in vivo.

It has been shown that many patients with aggressive malignancies spontaneously develop T cell responses to multiple tumor-associated Ags with time (54, 55). However, the effectiveness of such T cells is highly dependent on their activation/differentiation, persistence, and interaction with other cell populations that may initiate tumor regression and/or induce tumor tolerance and progression in vivo (5, 6, 13, 24, 56–61). In this study, we show that Ag-specific Tc1 effector cells not only induced an accumulation of highly differentiated CD4/$CD44^{high}$ and CD8/$CD44^{high}$ T cell subpopulations, but also enhanced their persistence and cell frequencies within the primary tumor environment. Conversely, such Tc1 effector cells markedly delayed the appearance of endogenous CD4/$CD44^{low}$ and CD8/$CD44^{low}$ T cells. Moreover, such nondifferentiated TIL cell subpopulations, elicited by adoptively transferred Tc1 cells, showed enhanced levels of acute activation as defined by coexpression of surface markers associated with TCR engagement (CD69) and early T cell activation (CD25). This suggested that type 1 CD8-mediated antitumor responses can preferentially influence T cell infiltration, localization, and cell numbers of select CD4 and CD8 T cell subpopulations and perhaps facilitate the differentiation (i.e., $CD44^{low}$ to $CD44^{high}$) and accumulation of more responsive TIL cell subpopulations at sites of mammary tumor growth.

Although CD25 is generally regarded as an activation marker, the elevated numbers of endogenous differentiated and nondifferentiated CD4 and CD8 cells coexpressing CD25 and CD69 may indicate the emergence of phenotypically diverse immunoregulatory T cell subpopulations at various stages of disease progression. Such cells, undergoing acute activation via TCR-mediated mechanisms, may be responsible for promoting and maintaining active T cell homeostasis that may directly and/or indirectly affect adoptively transferred Tc1 effector cell persistence and function in vivo. Moreover, such endogenous T cell subpopulations may be responsible for recruiting other functionally diverse immune cell repertoires within the tumor environment that may participate in promoting and enhancing T cell-mediated type 1 antitumor responses. Alternatively, it has been demonstrated that select regulatory T cell subpopulations, manifesting the phenotype CD3/CD4/CD25 (9, 10, 62, 63), are immunosuppressive and promote ineffective and/or transient antitumor responses. The elevated numbers of such TIL cell subpopulations observed in our studies may indicate the existence of intrinsic immune responses that promote local immunosuppression and/or aid in inducing or maintaining immune tolerance to progressing tumor growth (9–12, 62–67). We are currently investigating whether the removal and/or selective ablation of such acutely activated endogenous T cell subpopulations will directly and/or indirectly affect Tc1 effector cell fate, persistence, and function and/or modulate endogenous CD8-mediated type 1 antitumor responses to effectively regulate mammary tumor progression following Tc1 effector cell transfer.

Chemokines, such as IP-10, have been shown to play distinct roles in the regulation of local immune responses by influencing the balance between proinflammatory and anti-inflammatory T cells through specific cellular recruitment mechanisms (44–47). Moreover, IP-10 chemokine expression has not only been shown to be preferentially regulated by local IFN-$\gamma$ production, but also to enhance tumor regression by promoting type 1-like tumor immunity via Th1 or Tc1 cell recruitment (41–45, 68, 69). In this study, we show that IP-10 production by endogenous T cells, following administration of IFN-$\gamma$-producing CD8 effector cells, was markedly up-regulated at both early and late stages following Tc1 cell transfer. Moreover, such up-regulation of T cell-derived IP-10 within the tumor environment appeared to correlate with the appearance and accumulation of more differentiated and/or acutely

activated endogenous T lymphocyte subpopulations. It is interesting to speculate that the emergence of such endogenous T cell subpopulations and their capacity to produce IP-10 may further orchestrate and/or facilitate tumor infiltration by various T cell repertoires that may further promote type 1 CD8-mediated antitumor responses. Alternatively, IP-10 has been shown to profoundly affect tumor vascularization in a dose-dependent manner (41, 43, 69). The late emergence of such endogenous IP-10-producing T cell populations may represent regulatory T cells that use chemokine production to subsequently diminish tumor vascularization that may directly affect either tumor cell growth or keep effector T cell infiltration, function, or survival limited at later stages of tumor progression. Overall, our observations suggest that Ag-specific type 1 CD8-mediated antitumor responses are initially dependent, in part, on the following: 1) the interactions between donor CD8 T cell-derived IFN-$\gamma$ and select cytokine-inducible chemokines, such as IP-10; and 2) contributions by endogenous chemokine-secreting T cells and their quantitative, spatial, and/or temporal patterns of chemokine expression within the tumor environment. Such interactions may potentially influence CD8-mediated type 1 antitumor responses by modulating T cell recruitment, persistence, fate, and function within the tumor environment in vivo.

Collectively, these studies show that adoptive T cell transfer with tumor-reactive Tc1 effector cell subpopulations cannot only promote the accumulation of endogenous CD4 and CD8 TIL cell subpopulations, but also facilitate and preferentially affect their localization kinetics, activation/differentiation, and function within the primary tumor environment at various stages of tumor growth. This may aid, in part, in enhancing endogenous T cell responses and their cytokines/chemokines that effectively modulate antitumor responses to aggressive breast malignancies and offer insight into potential mechanisms for enhancing T cell immunotherapy in breast cancer patients.

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## Disclosures

The authors have no financial conflict of interest.

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