

The Antitumor Efficacy of IL2/IL21-Cultured Polyfunctional Neu-Specific T Cells Is TNF α /IL17 Dependent

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Abstract

Purpose: Infusion of HER2-specific T cells, derived from vaccine-primed patients and expanded with IL2/IL12, has induced tumor regression in a minority of patients with metastatic treatment-refractory HER2⁺ breast cancer. We questioned whether alteration of cytokine growth factors used to culture vaccine-primed T cells could improve antitumor activity.

Experimental Design: Using the TgMMTV-neu murine mammary tumor model, we cultured T cells derived from mice immunized with a previously defined neu class II peptide, p98-114 (neu p98), and evaluated different cytokine combinations for expansion.

Results: Infusion of neu p98-specific T-cell lines derived from all cytokine conditions evaluated resulted in significant antitumor activity compared with infused naïve splenocytes ($P < 0.05$). T cells cultured with IL2/IL21 could uniquely mediate complete regression of spontaneous mammary tumors. IL2/IL21 cultured

neu-specific T cells demonstrated a different cytokine secretion pattern as compared with other cultured T cells; secreting high levels of TNF α and IL17 ($P < 0.05$). Moreover, tumor-infiltrating CD8⁺ cells were significantly increased after the infusion of IL2/IL21 cultured T cells as compared with tumors treated with T cells expanded under other cytokine conditions ($P < 0.001$). The antitumor effect of the infusion of IL2/IL21 cultured cells was mediated by CD8 T cells. Depletion of TNF α or IL17, but not IFN γ , abrogated the tumor growth inhibition induced by the IL2/IL21 T cells and markedly decreased the influx of CD8 into tumors. Finally, IL2/IL21-cultured human antigen specific T cells also displayed a similar polyfunctional Th1/Th17 phenotype.

Conclusions: Expansion of HER2 vaccine-primed T cells with IL2/IL21 may have the potential to effectively mediate tumor regression when used in adoptive transfer. *Clin Cancer Res*; 22(9):2207–16. ©2015 AACR.

Introduction

Adoptive T-cell therapy has shown clinical efficacy in patients with advanced melanoma, but has not been as effective in other solid tumors. Efforts to improve the clinical efficacy of T-cell transfer have focused on chimeric antigen receptor (CAR)-engineered T cells, which have been enhanced to improve recognition of and interaction with tumor. Several hurdles have been identified in the clinical application of CAR to the treatment of solid tumors; the T cells can be short-lived, high-avidity CAR can interact with normal tissue, resulting in off-target effects and autoimmunity, and the T cells may become inactivated in the tumor microenvironment (1).

An alternative method of adoptive T-cell transfer is to use the patients' autologous T cells. Studies performed using autologous tumor-infiltrating lymphocytes (TIL) in melanoma demonstrated a 50% response rate in patients with metastatic disease (2). Often, TIL are not available due to inability to access metastatic sites, for this reason vaccine-primed T cells can be generated via active immunization, collected, and expanded *ex vivo* for therapeutic infusion. Antigen-specific T cells have been expanded after immunization to increase specificity for hTERT, survivin, MAGE-3, and HER2 and have shown some clinical benefit (3–5). The clinical efficacy of autologous T-cell infusions is hampered by the generation of lower avidity T cells, which slowly expand *in vivo* and also become inactivated in the immunosuppressive tumor microenvironment.

We questioned whether the antitumor efficacy of *ex vivo*-expanded autologous vaccine-primed T cells could be modulated via the cytokine culture conditions used for expansion. A focus on CD4⁺ tumor-specific Th1 offers several advantages over other T-cell populations. First, tumor antigen-specific CD4⁺ Th1 cells may home to the tumor and the inflammatory cytokines they secrete, such as IFN γ , may modulate the tumor microenvironment. Th1 cytokines enhance the function of local antigen-presenting cells (APC) and augment endogenous antigen presentation (6). Increased processing of endogenous tumor cells results in epitope spreading, the development of an immune response to the multiple immunogenic proteins expressed in the tumor (7). In addition, by providing a robust CD4⁺ Th1 T-cell response, tumor-specific CD8⁺ T cells will be elicited and proliferate endogenously (8). Finally, antigen-specific CD4⁺ T cells would provide the

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Translational Relevance

Adoptive T-cell therapy with tumor-infiltrating lymphocytes, vaccine-primed T cells, or chimeric antigen receptor-engineered T cells has shown some evidence of clinical efficacy in a minority of solid tumor patients treated. Methods to optimize different modes of T-cell transfer are under exploration. Data shown here demonstrate that variation in the cytokines used to expand autologous vaccine-primed tumor-specific T cells affects the phenotype as well as antitumor activity of the cells. IL2/IL21 when used in culture will generate polyfunctional type I T cells that enhance the egress of CD8 T cells into the tumor via TNF α and IL17 secretion and result in complete resolution of established tumors in TgMMTV-neu mice. IL21, when used in the culture of human antigen-specific T cells, will induce a similar phenotype. Polyfunctional Th1/Th17 cells may have the potential to effectively induce tumor regression when used in adoptive transfer.

environment needed to enhance and sustain tumor-specific T-cell immune responses over time.

We evaluated a variety of cytokine combinations, all previously shown to have utility in T-cell culture, to determine whether specific cytokines could affect the phenotype and antitumor function of tumor-specific T-helper cells suitable for therapeutic infusion.

Materials and Methods

Mice and the syngeneic tumor cell line

TgMMTV-neu mice (strain name, FVB/N-TgN(MMTVneu)-202Mul), 6 to 10 weeks of age, were obtained from Charles River Laboratory and bred under pathogen-free conditions at the University of Washington (Seattle, WA) in compliance with Institutional Animal Care and Use Committee guidelines. The neu-expressing mouse mammary carcinoma (MMC) cell line has been previously described (9). MMC cells were maintained in RPMI/1-glutamine/HEPES medium (Mediatech), supplemented with 10% FCS (Gemini), 1% penicillin/streptomycin (Mediatech, Inc.), and 55 μ mol/L beta-mercaptoethanol (Gibco).

Generation of neu antigen-specific T cells

Female TgMMTV-neu mice (6–8 weeks) without palpable tumors were immunized s.c. 3 times (7–10 days apart) with 100 μ g of neu peptide 98-114 (RLRIVRGTQLFEDKYAL; neu p98) (Genemed Synthesis Inc.). neu p98 has been shown to be a native MHC II epitope of the rat neu protein in TgMMTV-neu mice (10). Complete and Incomplete Freund's were used as adjuvants, as previously described (11). Splenocytes were harvested 7 to 10 days after the last vaccine. For T-cell expansion, splenocytes were stimulated with 10 μ g/mL of neu p98 at 3×10^6 cells/mL in culture media (RPMI/1-glutamine/HEPES medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 55 μ mol/L beta-mercaptoethanol). The stimulated cells were treated with IL2 (10 U/mL) on day 4 and restimulated with 10 μ g/mL of neu p98 on day 7. Respective cytokines were added on days 9 and 13. Recombinant cytokines and their final concentrations were as follows: IL2 (10 U/mL), IL4 (50 U/mL), IL7 (10 ng/mL), IL12 (5 ng/mL), IL15 (5 ng/mL), IL18 (100 ng/mL), or IL21

(100 ng/mL). All cytokines, except IL2 (Hoffman-La Roche) and IL18 (MBL International), were purchased from PeproTech (Rocky Hill, NJ). T cells were stimulated with soluble anti-CD3 antibody (50 ng/mL; eBioscience) on day 19 and IL2 (30 U/mL) was added every 2 to 3 days afterward. For *in vivo* studies, T cells were infused 2 to 5 days after anti-CD3 activation.

Flow cytometry

Cultured T-cell lines were stained with fluorochrome-conjugated monoclonal antibodies against CD3 (1 μ g), gamma-delta TCR, CD4, CD8, CD19, NK1.1 (0.5 μ g each) for subset analysis (all antibodies from BD Bioscience). Cells were stained with FOXP3 Alexa488 (1 μ g), CD3 PerCP (1 μ g), and CD4 APC (0.5 μ g) for regulatory T cells (Treg) analysis according to the FOXP3 staining protocol (eBioscience). Data acquisition was performed on a FACS Canto flow cytometer (BD Biosciences) and was analyzed using the FlowJo software (TreeStar).

IFN γ ELISPOT

Cultured T-cell lines were plated at 2×10^5 per well into IFN γ precoated ELISPOT plates. neu p98 (10 μ g/mL) was added into the cells in the presence of naïve splenocytes from TgMMTV-neu mice as APC. CD3 antibody (100 ng/mL; clone 145-2C11; eBioscience) was used as a positive control. IGFBP-2 p251-265 (GPLEHLYSLHIPNCD; irrelevant peptide, 10 μ g/mL) and medium only served as negative controls. The plates were cultured at 37°C/5%CO $_2$ for 3 days, and colored IFN γ spots were developed and counted as previously described (11). Data were reported as corrected neu p98 antigen-specific spots (number of mean spots in antigen wells minus spots in irrelevant peptide wells) per 2×10^5 T cells (4–6 replicates). The IFN γ spots in medium only were similar to that in irrelevant antigen wells ($P > 0.05$), and each positive control gave maximal levels of spots.

Adoptive T-cell transfer

T cells (5×10^7 cells) were injected i.v. in TgMMTV-neu mice bearing palpable (100 mm 3) spontaneous mammary tumors. Tumor growth was monitored every 2 to 3 days with Vernier calipers, and calculated as the product of length \times width \times height \times 0.5236. Data on tumor growth were presented as the mean tumor size \pm SEM of 5 mice per group. For other experiments, each cultured T-cell line (10^7) was admixed with live MMC cells (10^6) and implanted s.c. into the dorsal flank of TgMMTV-neu mice. Control (naïve) T cells were derived from spleen preparations and infused at the same dose as neu p98 T cells.

Cytokine analysis

Supernatants from cultured mouse T cells were collected at the end of expansion and stored at -80°C . Quantification of secreted cytokines (IFN γ , TNF α , IL17, IL5, IL10, and IL13) was performed using a Milliplex kit (Millipore) on a Luminex instrument (Qiagen) according to the manufacturer's instructions. Using the instrument software, the concentrations for the standards and samples were determined by a 5-parameter logistic method based on the mean fluorescent intensity. Experiments were repeated twice. Cytokine concentrations are reported in pg/mL.

IHC staining

To evaluate tumor infiltrating CD8 $^+$ T cells, tumors were frozen in Tissue-Tek OCT and stored at -80°C . Frozen tumors were stained with rat anti-mouse CD8 (clone KT15; AbD Serotec) using

previously described methods (12). CD8⁺ cells and DAPI-stained nuclei were counted with a fluorescent microscope (Zeiss LSM 510 META, Carl Zeiss Microimaging Inc.). Five to 10 random microscopic fields per mouse were counted and the mean of positive cells per mouse obtained. The data were expressed as the mean number (\pm SEM) of positive cells per field, or % of positive cells over the total number of nuclei in the field for 5 mice/group.

In vivo cell subset depletions

Depletion of CD4, CD8, and B cells were performed by i.p. injection of monoclonal antibodies. Anti-CD8 (150 μ g; Clone 2.43), anti-CD4 (250 μ g; clone GK1.5), or anti-CD22 (100 μ g; clone CY34.1.2) antibodies (UCSF) were injected into tumor-bearing mice which had received infusion of IL2/IL21-expanded T cells. The mice received three consecutive injections of appropriate antibody in the first week, followed thereafter by two injections weekly until the end of the experiment. Tumor growth was measured as described above and presented as mean tumor growth (\pm SE) of 4 mice per group.

In vivo cytokine depletions

TgMMTV-neu mice bearing spontaneous tumors (100 mm³) were infused i.v. with 4×10^7 T cells (day 0) and then injected with 100 μ g neutralizing antibodies against IFN γ (clone 37895), or TNF α (clone MP6-XT22), or IL17 (clone 50104) i.p. on the following day and then every other day (days 1, 3, 5, 7, 9). Respective isotype control antibodies (IgG2a, clone 54447, for IL17 and IFN γ ; IgG1a, clone 43414, for TNF α) were administered to control mice in an identical fashion. All antibodies were purchased from R&D Systems, except for TNF α (BioLegend). The same number of naïve splenocytes was used as controls. Tumor growth was measured as described above and presented as mean tumor growth (\pm SE) of 3 mice per group. Experiments were repeated twice.

Gene expression analysis

To evaluate Granzyme B mRNA expression, spontaneous tumors were obtained from the mice 21 days after the infusions of neu p98-specific and naïve T cells. Total RNA was extracted from the expanded T cells using a RNA Easy Mini Extraction Kit (Qiagen), and the integrity of the RNA was confirmed using an Agilent BioAnalyzer. cDNA synthesis and real-time RT-PCR were performed as previously described (13) using primer and probes from Applied Biosystems. The levels of Granzyme B mRNA expression were normalized to β -actin using the $\Delta\Delta C_t$ method (13).

To evaluate Th1/Th17 gene expression, CMV-specific human T cells were expanded *ex vivo* with the different cytokine conditions of IL2 (Hoffman-La Roche), IL2/IL12 (R&D Systems), and IL2/IL21 (Peprotech) using previously published methods (14). Total RNA was extracted and real-time RT-PCR was performed as above. The levels of mRNA expression of Th1/Th17 cytokine (IFN γ , TNF α , IL17, and IL21) and Th17-differentiation genes RORc and IFN γ regulatory gene IRF-4 were normalized to β -actin.

Human IL17 and IFN γ ELISPOT

Peripheral blood mononuclear cells derived from a breast cancer patient after HER2 peptide vaccination were stimulated with three MHC class II epitopes (p369-384, p688-703, and p971-984; 10 μ g/mL) (15) and cultured with IL2/IL21 as

described above. The expanded T cells were plated at 1×10^5 /well in human IL17 and IFN γ precoated ELISPOT plates (Mabtech). The cells were restimulated with HER2 peptides loaded on autologous APC with no-antigen wells as control. After 48-hour culture, IFN γ spots were developed and counted as described (14), and IL17 spots were developed using a biotinylated IL17 antibody (Mabtech). Data are reported as mean spots per well of 5 replicates.

Statistical analyses

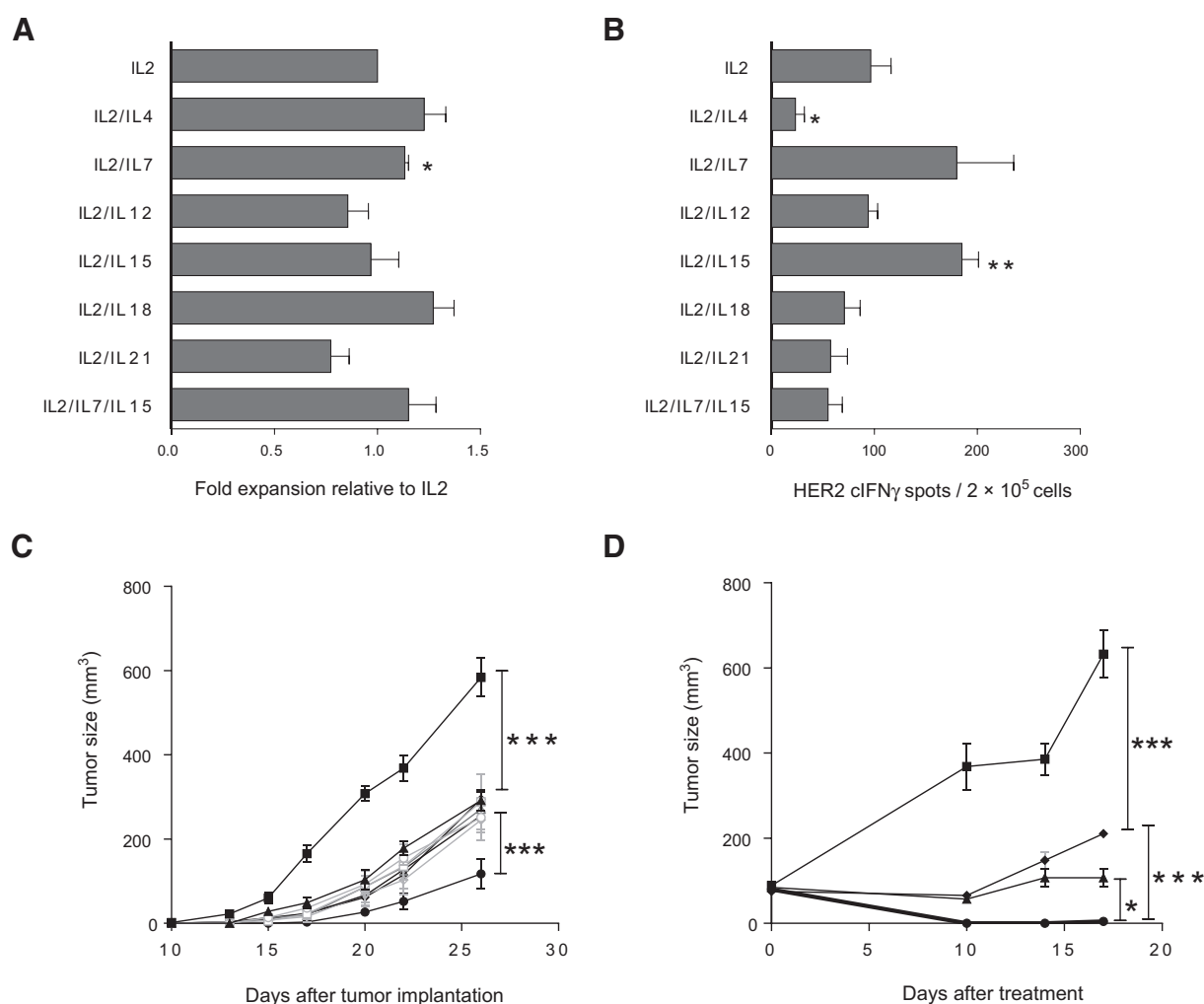
Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software). The unpaired, two-tailed Student *t* test was used to evaluate differences in tumor cell growth, T-cell responses in ELISPOT, cytokine production, numbers of tumor-infiltrated CD8⁺ lymphocytes, and gene expression. The two-way analysis of variance (ANOVA) test was used to compare the tumor growth in TgMMTV-neu mice among experimental groups. A value of $P < 0.05$ was considered statistically significant.

Results

Specific cytokines influence the number of antigen-specific T cells and antitumor activity generated

We evaluated several common cytokines based on their potential benefit for *ex vivo* T-cell expansion, including IL2 alone (2, 16, 17) or IL2 combined with IL4 (18), IL7 (19, 20), IL15 (20, 21), IL7/IL15 (20), IL12 (22), IL18 (23), or IL21 (24). The T-cell lines generated from all cultures were predominantly CD3⁺ (Supplementary Table S1A). Most T-cell lines had a higher percentage of CD8⁺ cells than CD4⁺ cells among the CD3⁺ T cells, except the IL2 and IL2/IL12-cultured cells that were predominantly CD4⁺. CD4⁺FOXP3⁺ Treg were rarely expanded in all cytokine conditions (Supplementary Table S1B). The cytokines evaluated had a minimal impact on the overall fold expansion of CD3⁺ T cells compared with IL2 alone, except for the IL2/IL7 culture condition ($P < 0.05$; Fig. 1A). All cytokine-conditioned T cells demonstrated a specific IFN γ response to neu p98 as compared with an irrelevant peptide derived from IGFBP-2 ($P < 0.05$; Supplementary Fig. S1). After correcting for the irrelevant peptide responses, only IL2/IL15-conditioned T cells generated significantly greater antigen-specific IFN γ responses relative to IL2 alone ($P < 0.01$; Fig. 1B). The culture condition using IL2/IL4 was the least successful in expanding antigen-specific T cells.

To test the effects of various cytokine culture conditions on the antitumor efficacy of neu p98-specific T cells *in vivo*, the cultured T cells from all conditions were admixed with live MMC cells and administered to TgMMTV-neu mice. As shown in Fig. 1C, the T-cell lines from all cytokine-cultured conditions significantly inhibited the tumor growth ($P < 0.001$; $n = 5$) as compared with naïve splenocytes. Notably, only the lines cultured with IL2/IL21 induced a significant tumor inhibition greater than IL2 alone and all other lines ($P < 0.001$). We then infused the cultured T cells into mice that had developed spontaneous mammary tumors. Similarly, the cultured T cells from selected cytokine conditions tested significantly inhibited the tumor growth ($P < 0.001$) as compared with naïve splenocytes. However, IL2/IL21-cultured T cells were significantly more effective at mediating tumor inhibition as compared with IL2 only ($P < 0.05$) and IL2/IL7/IL15 ($P < 0.001$, $n = 3$; Fig. 1D). Indeed, neu-specific T cells cultured in IL2/IL21 induced complete tumor regression.

**Figure 1.**

Specific cytokines influence the number of antigen-specific T cells and antitumor activity generated. A, cell growth under different cytokine conditions. Bars, mean fold expansions relative to IL2 condition for all cytokine conditions. *, $P < 0.05$. B, neu p98-specific IFN γ secretion from T cells cultured under different cytokine conditions. *, $P < 0.05$; **, $P < 0.01$ vs. IL2 only. Data, representative of two experiments. C, tumor growth (y-axis) over time (x-axis) in TgMMTV-neu mice implanted with MMC admixed with naïve splenocytes (-■-) or with T-cell lines expanded in IL2 (-▲-), or combined with IL4 (-O-), IL7 (-□-), IL12 (-△-), IL15 (-▽-), IL18 (-◇-), IL21 (-●-), or IL7/IL15 (-◆-). Data points, mean (\pm SE) of tumor size from five mice. D, tumor growth (y-axis) over time (x-axis) of spontaneous tumors developed in TgMMTV-neu mice treated with naïve splenocytes (■) or with T-cell lines expanded in IL2 (▲), or combined with IL21 (●), or IL7/IL15 (◆). Data points, mean (\pm SE) of tumor size from 3 mice. *, $P < 0.05$; ***, $P < 0.001$.

T cells expanded with IL2/IL21 demonstrate a polyfunctional Th1/Th17 phenotype

To further explore the potential differences in the T-cell lines that could be contributing to the variation in antitumor efficacy, we evaluated antigen-specific cytokine secretion. As shown in Fig. 2A, the levels of IFN γ from IL2/IL12, IL2/IL18, and IL2/IL21 conditions were significantly increased as compared with IL2 alone ($P < 0.05$). The levels of TNF α from all cytokine conditions were significantly greater than IL2 alone ($P < 0.05$). Notably, the level from the IL2/IL21 culture condition was strongly enhanced nearly 2-fold above all other conditions (Fig. 2B). Only IL2/IL21-cultured T cells showed significantly greater levels of IL17 than IL2 alone ($P < 0.05$) and all other culture conditions (vs. IL2/IL12, IL2/IL15, IL2/IL18, IL2/IL7/IL15, IL2/IL7, or IL2/IL4, $P < 0.05$; Fig. 2C). Moreover, IL2/IL21-conditioned T cells secreted significantly less IL5 than IL2/IL7, IL2/IL15, IL2/IL18, and IL2/IL7/IL15 ($P <$

0.05; Fig. 2D), and secreted significantly less IL10 than IL2/IL4, IL2/IL12, IL2/IL15, IL2/IL18, and IL2/IL7/IL15 ($P < 0.05$; Fig. 2E), although these were not significantly less than IL2 alone. In addition, IL2/IL21-cultured T cells secreted significantly less IL13 than IL2 alone ($P < 0.05$) and all other cytokine conditions (vs. IL2/IL12, or IL2/IL18, $P < 0.05$; vs. IL2/IL7, IL2/IL15, or IL2/IL7/IL15, $P < 0.01$) except versus IL2/IL4 (Fig. 2F). Thus, T cells expanded in IL2/IL21 exhibited a unique Th1/Th17 cytokine profile when compared with other culture conditions.

The antitumor efficacy of IL2/IL21 neu-specific T cells is mediated by CD8⁺ T cells

We evaluated the frequencies of tumor-infiltrating CD8⁺ T cells after the infusion of IL2, IL2/IL21, IL2/IL7/IL15-cultured and naïve T cells in mice bearing spontaneous tumors. As shown in Fig. 3A, the number of CD8⁺ T cells in all

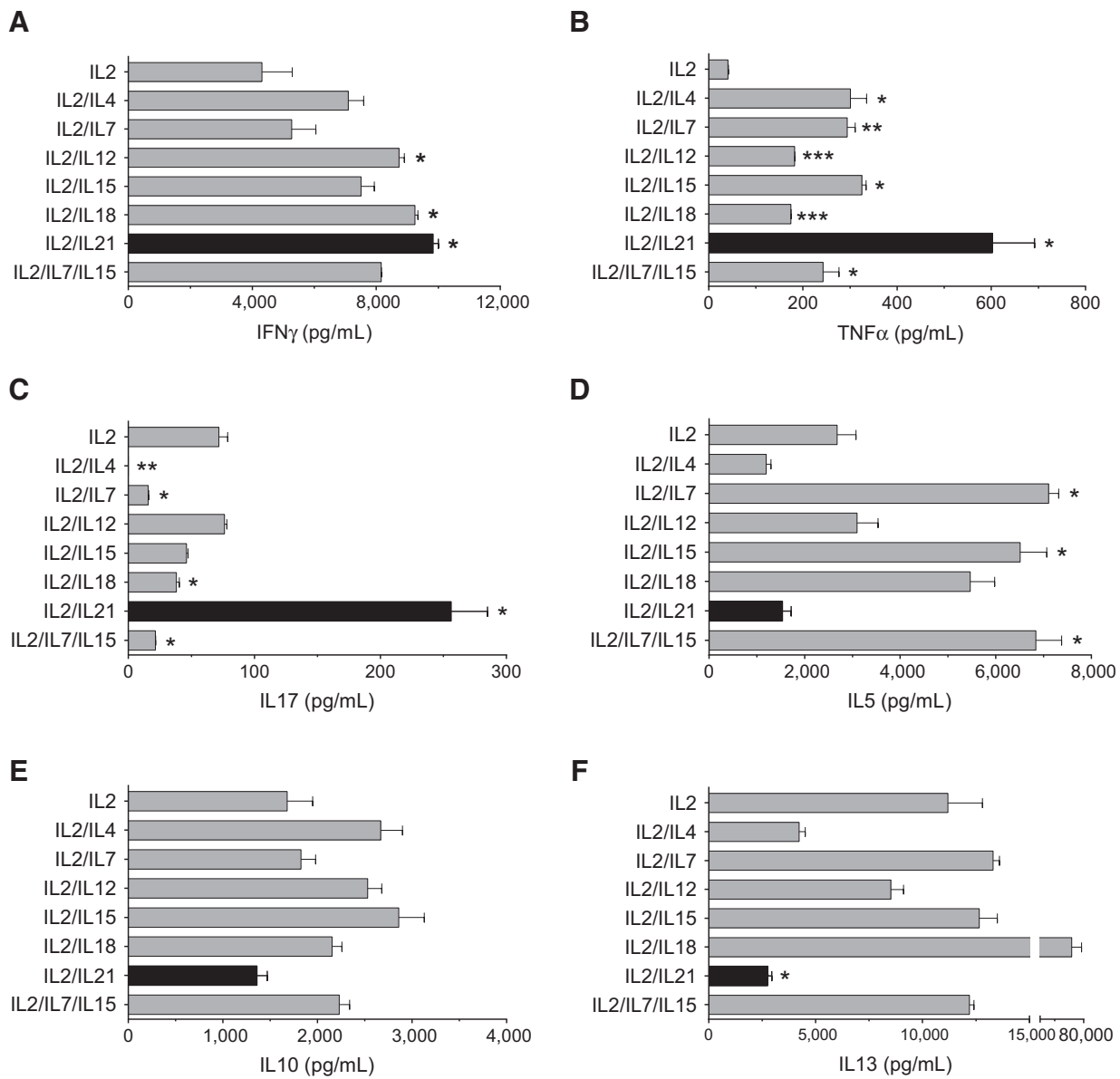


Figure 2. T cells expanded in IL2/IL21 condition demonstrate a polyfunctional Th1/Th17 phenotype. Bars, mean (\pm SE) of duplicates for all culture conditions. A, IFN γ . (B) TNF α , (C) IL17, (D) IL5, (E) IL10, (F) IL13. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. IL2. Results shown are representative of two experiments.

cytokine-cultured T-cell infused were significantly higher than that in naïve T-cell infused (mean \pm SE: naïve, 0.2 ± 0.2 ; IL2, 2.4 ± 0.5 , $P < 0.01$; IL2/IL21, 26.6 ± 1.0 , $P < 0.001$; IL2/IL7/IL15, 7.4 ± 0.9 , $P < 0.001$; $n = 5$). However, the tumor-infiltrating CD8⁺ cells in mice infused with IL2/IL21-cultured T cells were significantly increased compared with that in mice infused with IL2 cultured T cells ($P < 0.001$) and IL2/IL7/IL15-cultured T cells ($P < 0.001$). Supplementary Fig. S2 shows a representative IHC staining for intratumoral CD8⁺ cells. We further analyzed the mRNA expression of Granzyme B within the spontaneous tumors after the T-cell infusions. As shown in Fig. 3B, the expression was significantly higher in p98-specific T cells infused versus naïve T cell-infused mice (p98

T cells, 0.002 ± 0.000 ; naïve T cells, 0.001 ± 0.000 ; $n = 2$; $P < 0.05$).

To determine which immune cell subsets mediated the antitumor effect of IL2/IL21-cultured T cells, we then selectively depleted CD4⁺, CD8⁺ T cells, or CD22⁺ cells after the infusion of the T cells into TgMMTV-neu mice with spontaneous tumors. As shown in Fig. 3C, the elimination of CD8⁺ T cells in mice significantly hindered the antitumor efficacy of IL2/IL21-cultured neu p98-specific T cells (mean \pm SE: T cells only, 110 ± 10 , vs. with CD8 depletion, 371 ± 53 ; $P < 0.001$), whereas the depletion of either CD4⁺ T cells (116 ± 31 ; $P > 0.05$) or CD22⁺ B cells (101 ± 19 , $P > 0.05$) demonstrated no impact.

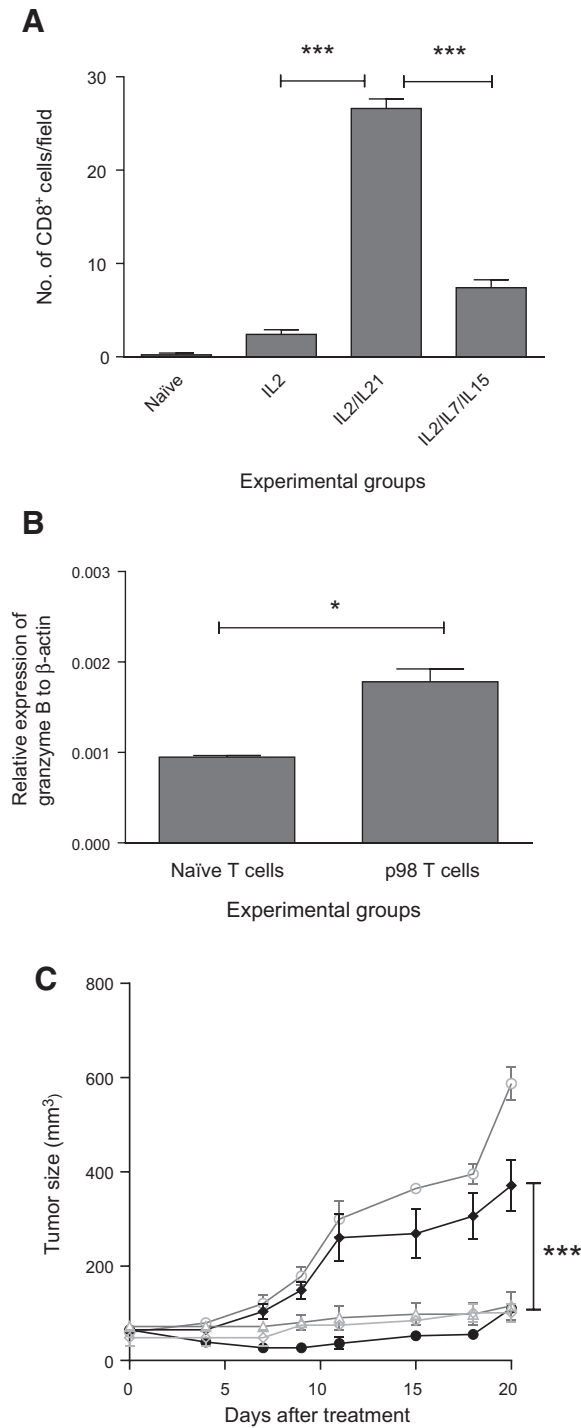


Figure 3. The antitumor efficacy of IL2/IL21 neu-specific T cells is mediated by CD8⁺ T cells. A, quantitation of CD8⁺ cells in tumors after infusion of IL2, IL2/IL21, or IL2/IL17/IL15-cultured p98 T cells, or naive T cells. Columns and bars represent the mean (\pm SE) of 5 mice per group. **, $P < 0.01$; ***, $P < 0.001$. B, relative mRNA expression of Granzyme B to β -actin. Bars are the mean (\pm SE) of 2 mice per group, 3 replicates per mouse. *, $P < 0.05$. C, spontaneous tumors developed in TgMMTV-neu mice treated with naive splenocytes (-○-), IL2/IL21-cultured T cells only (-●-), or with anti-CD8 (-◆-), or with anti-CD4 (-△-), or anti-CD22 antibody (-◇-). Data points are the mean of tumor size (\pm SE) from 4 mice. ***, $P < 0.001$.

The antitumor efficacy of IL2/IL21-cultured neu-specific T cells is IL17 and TNF α dependent

As IL2/IL21-cultured neu p98-specific T cells demonstrated a unique cytokine profile and secreted high levels of IFN γ , TNF α , and IL17, we questioned whether the therapeutic efficacy of the T cells was diminished when these cytokines were blocked *in vivo*. The respective neutralizing cytokine antibodies or isotype controls were administered to TgMMTV-neu mice with spontaneous breast tumors 24 hours after the infusion of IL2/IL21-cultured T cells. We found that neutralization of TNF α significantly impaired T-cell efficacy, resulting in increased tumor volumes compared with control (mean \pm SE of tumor size: IL2/IL21 only, 61 ± 12 ; vs. with TNF α Ab, 491 ± 28 ; $P < 0.001$; Fig. 4A). Furthermore, the neutralization of IL17 abolished the antitumor efficacy elicited by IL2/IL21-cultured T cells (572 ± 7 ; $P < 0.001$; Fig. 4B). However, the neutralization of IFN γ had little impact on the antitumor effect elicited by the T cells (104 ± 6 ; $P > 0.05$; Fig. 4C). As expected, all isotype controls had no effect on the antitumor response ($P > 0.05$; Supplementary Fig. 4A–4C). To determine whether the unique cytokine secretion pattern of neu-specific T cells cultured in IL2/IL21 contributed to enhanced CD8⁺ T-cell egress into the tumors, we examined the number of tumor-infiltrating CD8⁺ T cells after the neutralization of TNF α , IL17, or IFN γ , respectively. Blocking any cytokine secretion resulted in a significant reduction in the number of tumor-infiltrating CD8⁺ T cells compared with isotype control (mean \pm SE of % of CD8⁺ T cells: control: 0.14 ± 0.00 , vs. TNF α , 0.05 ± 0.00 , $P < 0.01$; vs. IL17, 0.02 ± 0.00 , $P < 0.001$; and vs. IFN γ , 0.09 ± 0.01 , $P < 0.05$). However, both TNF α and IL17 neutralization resulted in a significantly greater reduction of CD8⁺ T cells within the tumor compared with IFN γ neutralization ($P < 0.01$ and $P < 0.001$ respectively; Fig. 4D).

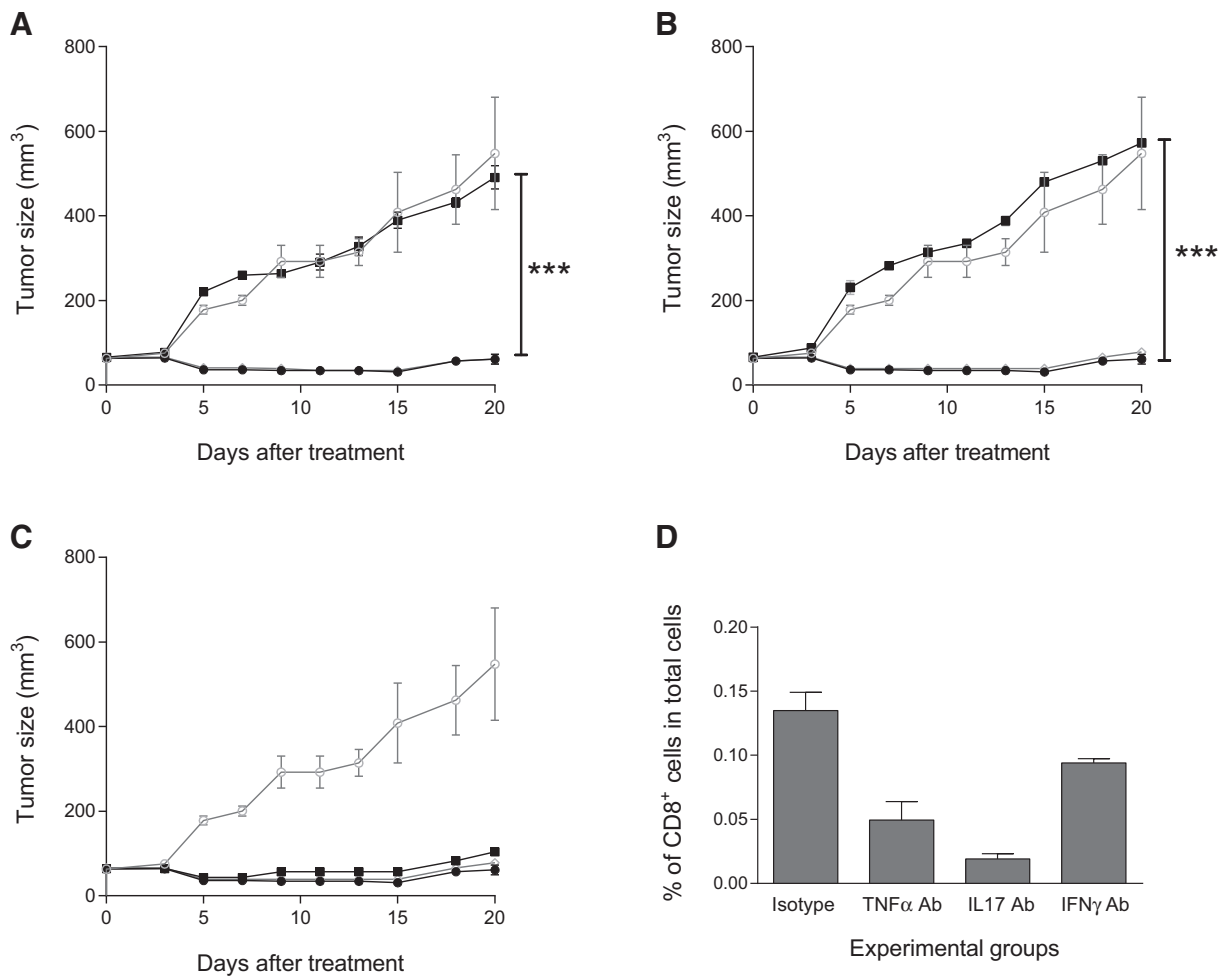
The expression of Th1/Th17 differentiation genes is increased in IL2/IL21-cultured CMV-specific T cells

We cultured human CMV-specific T cells with IL2, IL2/IL12, and IL2/IL21 to determine whether a similar phenotype could be induced. T cells from a CMV⁺ donor cultured in IL2/IL21 showed a significant increase not only in IFN γ (Fig. 5A, $P < 0.01$) but also in IL17 (Fig. 5B, $P < 0.01$), IL21 (Fig. 5C, $P < 0.01$) and IRF-4 (Fig. 5D, $P < 0.001$) gene expression as compared with the other two culture conditions. In addition, expression of ROR γ was significantly increased versus IL2, $P < 0.05$; versus IL2/IL12, $P = 0.053$ (Fig. 5E). Human T cells cultured in IL2/IL21, however, demonstrated less TNF α expression than IL2/IL12-cultured cells (Fig. 5F; $P < 0.01$).

We further examined whether polyfunctional human HER2-specific T cells can be generated from a patients who had received a HER2 peptide-based vaccine (15). The IL2/IL12-cultured T cells were restimulated with HER2 peptide antigens with no added antigen as a control. As shown in Supplementary Fig. S3, both antigen-specific IFN γ (HER2, 49 ± 4 , no antigen, 1 ± 1 ; $n = 5$; $P < 0.001$) and IL17 (HER2, 30 ± 4 ; no-antigen, 9 ± 2 ; $n = 5$; $P < 0.01$) secretion were significantly higher than controls.

Discussion

We used a single class II-restricted epitope derived from the tumor antigen neu, p98-114, to vaccinate mice and generate neu-specific T cells to expand under different culture conditions. Our goal was to determine the role of cytokines in influencing the

**Figure 4.**

The antitumor efficacy of IL2/IL21-cultured neu-specific T cells is IL17 and TNF α dependent. A–C, spontaneous tumors developed in TgMMTV-neu mice after infusion with naïve splenocytes (–○–), or with IL2/IL21-cultured T cells alone (–●–), or with isotype control (–◇–), or neutralizing anti-IL17 (A), or TNF α (B), or IFN γ (C) antibodies (–■–). Data points, mean tumor size (\pm SE) of three mice. Data are representative of 2 separate experiments. ***, $P < 0.001$. D, number of CD8⁺ cells % of total cells (y-axis) in isotype control, and TNF α , IL17 or IFN γ -depleted mice after T-cell infusion (x-axis). Columns and bars, mean (\pm SE) of 5 mice per group. ***, $P < 0.001$.

phenotype and enhancing the antitumor efficacy of autologous vaccine-primed tumor-specific T cells. Studies described here demonstrate that IL2/IL21, when used to culture either murine or human antigen-specific T cells, will induce a polyfunctional T-cell phenotype, Th1/Th17. Moreover, the IFN γ -, TNF α -, IL17-secreting cells generated in mice demonstrate potent antitumor activity mediated by CD8 T cells. Finally, TNF α and IL17 were necessary for the antitumor response, and secretion of these cytokines was associated with enhanced CD8 T-cell egress into mammary tumors.

Polyfunctional T cells are more effective in eradicating tumors or viruses than T cells that secrete a single dominant cytokine (25, 26). Antigen-specific polyfunctional T cells can amplify inflammation in the tumor microenvironment and, via diverse cytokine secretion, stimulate cross-priming by activating APC. The development of polyfunctional T cells is associated with clinical response after cancer immunotherapy. The combination of a TLR5 ligand with a PI3K inhibitor resulted in the generation of polyfunctional T cells secreting IFN γ , IL17, and IL2 (27). In

multiple mouse models, the antitumor effect of this drug combination was associated with increased levels of these polyfunctional T cells. Polyfunctional T cells induced by CTLA-4 blockade and secreting IFN γ , MIP-1beta, and TNF α were found in increased frequency in responding patients with metastatic melanoma as compared with nonresponders (28). Most cytokine conditions we tested generated a neu p98-specific T-cell population with a mixed phenotype of type I and type II cells. Although IFN γ and TNF α were secreted at greater levels than cells generated with IL2 alone, only the cells cultured with IL21 significantly secreted IL17. Moreover, secretion of type II cytokines was markedly diminished with this culture condition. IL21 is an autocrine growth factor for Th17 cells (29). We show that human Th1/Th17 T cells can also be elicited with IL2/IL21 culture. Not only is the specific cytokine gene expression enhanced after culture, but the Th17 transcription factor, RORc and IFN γ regulatory gene, IRF-4, were also increased. Although TNF α expression was not as elevated as in the mice, further refinement of the cytokine culture conditions could enhance TNF α secretion. The ability to recapitulate the

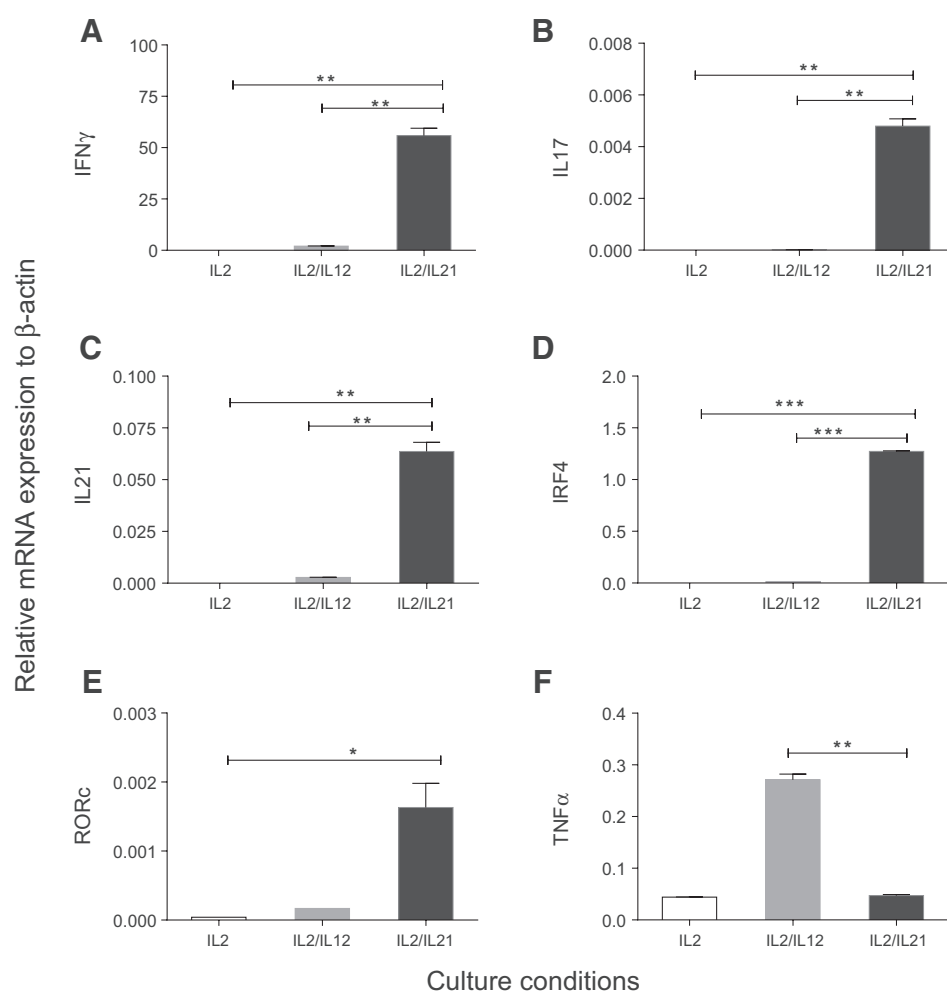


Figure 5. The expression of Th1/Th17 differentiation genes is increased in IL2/IL21-cultured CMV-specific T cells. Relative mRNA expression to β -actin (y-axis) in IL2 (white columns), IL2/IL12 (gray), and IL2/IL21 (black) cultured condition (x-axis). A, IFN γ , (B) IL17, (C) IL21, (D) IRF-4, (E) RORc, and (F) TNF α . Bars are the mean (\pm SE) of duplicates for all culture conditions. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

polyfunctional phenotype with human antigen-specific T cells provides the basis for the potential therapeutic infusion of Th1/Th17 tumor-specific T cells. Th17 cells have been shown to have "stem cell-like" properties (30) and have increased potential for proliferation and self-renewal (31). Thus, polyfunctional IL17-secreting T cells may persist as effector memory cells much longer than other forms of adoptively transferred cells, such as CAR, which can be short-lived (32). The ability of these cells to induce the same type of response across other murine models has yet to be determined. For example, in highly inflammatory cancer, such as colon cancer arising from colitis, IL17 secretion can induce further proliferation of the tumor and increase cell motility, thus enhancing tumor growth (33, 34).

CD8 T cells are important effectors in the elimination of human malignancy. High levels of tumor-infiltrating CD8 T cells have been found to be associated with a survival benefit in many different types of cancers (35–37). Especially in breast cancer, CD8 TIL have been shown to be an independent predictor of pathologic complete response after chemotherapy (38). Most breast cancers, however, have low to no CD8 TIL. This is most likely due to the Th2 bias that occurs early in oncogenesis, resulting in significant antibody generation but low type I T-cell expansion (39–41). We show that Th1/Th17-secreting T cells generated with IL2/IL21 drive significantly more CD8 T cells into

the tumor after infusion than cells cultured with more standard cytokine combinations. These data support the notion that the cytokine phenotype of CD4 T cells is a critical factor in the effective function of CD8 cytotoxic T cells. Investigations in both infectious disease and cancer have underscored the role of CD4 Th. IFN γ -secreting CD4⁺ T cells have been shown to activate dendritic cells, resulting in the generation of CMV-specific CD8⁺ T cells (42). *In vitro* studies demonstrate antigen-specific CD4 are required to effectively stimulate functional CMV-specific cytotoxic T cells. In cancer, specifically in the neu model, Th1 cells secreting IFN γ were essential for mediating tumor regression by inducing MHC expression on tumor cells, thereby enhancing direct recognition by both CD8 and CD4 T cells (43). The neutralization of IL17 and TNF α markedly reduced CD8 TIL. This observation suggests that these cytokines are uniquely associated with tumor CD8 T-cell recruitment.

Increasing CD8 T-cell influx into tumors has been a barrier to successful adoptive T-cell therapy in solid tumors, including breast cancer. The ability of antigen-specific T cells to home to tumors is dependent on a network of receptors and secreted proteins; selectins, integrins, and chemokines expressed both on the T cell as well as on or by tumor cells or other immune system cells (44). Type I cytokines have been shown to induce the upregulation of integrin receptors or the production of

chemokines. Thus, the generation of antigen-specific T cells secreting specific cytokines can allow direct modulation of the tumor microenvironment to enhance CD8 T-cell influx into the tumor. Recent studies have demonstrated that IL17 and TNF α act synergistically to induce the expression of both P- and E-selectin on endothelium (45). The two cytokines also enhance endothelial expression of specific chemokines, such as CXCL2 and CXCL5, significantly increasing T-cell transmigration into inflammatory sites (45). Th17 cells have been shown to be able to recruit effector T cells to the tumor via stimulation of CXCL9 and CXCL10 as well as directly activate CD8 T cells by recruitment of specific dendritic cell populations (46, 47). The importance of IL17 on T-cell recruitment is underscored by experiments demonstrating that the antitumor effects of Th17 cells are abrogated in CCR6-deficient mice (47). CCR6 regulates the recruitment of T cells to sites of an active immune response. Polyfunctional tumor-specific T cells that secrete both IL17 and TNF α may be ideally suited for use in adoptive T-cell transfer for the treatment of malignancy. Moreover, whether polyfunctional T cells will affect the expression of PD-L1 on tumor and antigen-presenting cells over time could influence the role of immune checkpoint inhibitors in further augmenting the antitumor immunity induced by these cells.

In summary, the choice of cytokine combination to expand autologous tumor-specific T cells is critically important in affecting the antitumor function of those cells. Specifically, the generation of Th1/Th17 polyfunctional T cells with IL2 and IL21 and other cytokines may result in a greater clinical response rate and potentially more sustained or durable responses due to the impact of these cytokines on CD8 T-cell tumor infiltration.

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Disclosure of Potential Conflicts of Interest

M.L. Disis reports receiving commercial research grants from EMD Serono, Seattle Genetics, and VentiRx; and holds ownership interest (including patents) in Epithany, UW Patents, and VentiRx. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Phan-Lai, M.L. Disis
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