In Vivo Ligation of CD40 Enhances Priming Against the Endogenous Tumor Antigen and Promotes CD8+ T Cell Effector Function in SV40 T Antigen Transgenic Mice

Kevin Staveley-O'Carroll, Todd D. Schell, Marcela Jimenez, Lawrence M. Mylin, M. Judith Tevethia, Stephen P. Schoenberger and Satvir S. Tevethia

*J Immunol* 2003; 171:697-707; doi: 10.4049/jimmunol.171.2.697

http://www.jimmunol.org/content/171/2/697

---

**References**

This article cites 72 articles, 41 of which you can access for free at:

http://www.jimmunol.org/content/171/2/697.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
In Vivo Ligation of CD40 Enhances Priming Against the Endogenous Tumor Antigen and Promotes CD8⁺ T Cell Effector Function in SV40 T Antigen Transgenic Mice

Kevin Staveley-O’Carroll,*† Todd D. Schell,* Marcela Jimenez,‡ Lawrence M. Mylin,2* M. Judith Tevethia,* Stephen P. Schoenberger,‡ and Satvir S. Tevethia3*

The ability to initiate and sustain CD8⁺ T cell responses to tumors in vivo is hindered by the development of peripheral T cell tolerance against tumor-associated Ags. Approaches that counter the onset of T cell tolerance may preserve a pool of potentially tumor-reactive CD8⁺ T cells. Administration of agonist Ab to the CD40 molecule, expressed on APCs, can enhance immunization approaches targeting T lymphocytes in an otherwise tolerance-prone environment. In this report, the effects of anti-CD40 administration on priming of naive CD8⁺ T cells against an endogenous tumor Ag were investigated. Line 501 mice express the SV40 large T Ag oncoprotein as a transgene from the α-amylase promoter, resulting in the development of peripheral CD8⁺ T cell tolerance to the H-2-Db-restricted immunodominant epitope I of T Ag by 6 mo of age, before the appearance of osteosarcomas. We demonstrate that naive epitope I-specific TCR transgenic (TCR-I) T cells undergo peripheral tolerance following adoptive transfer into 6-mo-old 501 mice. In contrast, administration of agonistic anti-CD40 Ab led to increased expansion of TCR-I T cells in 501 mice, the acquisition of effector function by TCR-I T cells and the establishment of T cell memory. Importantly, this enhanced priming effect of anti-CD40 administration did not require immunization and was effective even if administered after naive TCR-I T cells had encountered the endogenous T Ag. Thus, anti-CD40 administration can block the onset of peripheral tolerance and enhance the recruitment of functionally competent effector T cells toward an endogenous tumor Ag. The Journal of Immunology, 2003, 171: 697–707.

Due to their specificity and potent effector function, CD8⁺ T cells are an attractive target population for immunotherapeutic approaches to cancer. Many tumor-associated Ags recognized by CD8⁺ T cells have now been identified and represent nonmutated self-Ags that can serve as the focus for vaccination approaches to cancer (1, 2). Several mechanisms have been identified, however, that might limit the effectiveness of targeting self-reactive T cells, including deficiencies in the T cell repertoire due to negative selection of potentially tumor Ag-reactive T cells (3–6) as well as regulatory mechanisms that downmodulate immune responses to self-Ags in the periphery (7). In particular, recent evidence suggests that the recognition of peripheral self-Ags by naive CD8⁺ T cells leads to activation, expansion, and subsequent deletion of these self-reactive T cells to maintain tolerance to self-Ags in the periphery (8). Thus, strategies that protect naive tumor-reactive T cells from destruction or lead to the expansion and maintenance of effector and memory T cells within the tumor-bearing host will be critical for designing immunotherapeutic approaches to cancer.

Mice that develop spontaneous tumors due to the transgenic expression of oncogenes provide realistic models to assess immunotherapeutic strategies for potential use in the treatment of human cancer. Expression of the large tumor Ag (T Ag) from SV40 as a transgene in mice leads to the development of spontaneous tumors (9, 10). This oncogenic activity stems from the ability of the T Ag to inactivate the tumor suppressor proteins Rb and p53 as well as initiate cell cycle progression (11). In addition, T Ag serves as the target of a strong CTL response that can lead to the rejection of T Ag expressing tumors (12). Immunization of C57BL/6 mice with wild-type T Ag leads to the development of CTL specific for these three immunodominant epitopes: designated epitope I (T Ag residues 206–215), epitope II/III (T Ag residues 223–231), and epitope IV (T Ag residues 404–411) (13–15). Epitopes I and II/III are H-2-Db restricted, whereas epitope IV is H-2-Kk restricted.

Expression of T Ag as a transgene also can lead to the onset of either central or peripheral CD8⁺ T cell tolerance to the T Ag epitopes, depending on the site and timing of transgene expression (4, 16–19). Line B6/SV (H-2b) transgenic mice express T Ag under the α-amylase promoter, resulting in the expression of T Ag in the salivary glands and in bone (20, 21). Although neoplasia is not detected in the salivary glands, T Ag expression in bone leads to the development of osteosarcomas that can metastasize to the liver and lung. The expression of T Ag as a self Ag in 501 mice leads to the progressive loss of CTL responses to the T Ag epitopes (19). In particular, loss of CTL responsiveness to T Ag epitope I is tightly associated with the onset of T Ag expression in 501 mice.

* Abbreviations used in this paper: T Ag, large tumor Ag; DC, dendritic cell; LN, lymph node; NP, nucleoprotein; Flu, influenza virus; HA, hemagglutinin; rVV, recombinant vaccinia virus; TCR-I, epitope I-specific TCR transgenic.

Received for publication April 4, 2003. Accepted for publication May 6, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by research Grants CA-25000 (to S.S.T.) and CA-24694 (to M.J.T.) from the National Cancer Institute, National Institutes of Health, and the Franklin Martin, MD, FACS Memorial Faculty Research Award from The American College of Surgeons (to K.S.-O.), and a grant from the Pennsylvania State University Cancer Center (to K.S.-O.).

2 Current address: Department of Natural Sciences, Messiah College, Box 3030, Grantham, PA 17027.

3 Address correspondence to Dr. Satvir S. Tevethia, Department of Microbiology and Immunology, Pennsylvania State University College of Medicine, Hershey, PA 17033. E-mail address: sst1@psu.edu

Copyright © 2003 by The American Association of Immunologists, Inc.
Immunization of 501 mice at 6 mo of age, when T Ag is expressed at high levels in the salivary glands (21), failed to induce epitope I-specific CTL, although immunization of these mice at 3–4 mo of age resulted in the successful recruitment of epitope I-specific CTL (19). Whether the loss of epitope I-specific CTL activity in line 501 mice is due to the peripheral deletion of reactive CD8+ T cells or the induction of unresponsiveness is unknown.

The presence of potentially autoreactive cells in peripheral lymphoid organs can result in either ignorance of cognate Ag, unless a response is initiated by exogenous immunization (22–24), or Ag encounter resulting either in an immune response (25, 26) or the induction of tolerance (19, 27–30). It has become increasingly clear that the nature of the immune response to peripheral Ag is largely determined by the characteristics of the APC (31). Both T cell activation and the induction of peripheral T cell tolerance can be mediated through recognition of Ag on bone marrow-derived APCs, which are most likely dendritic cells (DCs) (28, 32–34). Recent evidence suggests that tissue-resident DCs with an immature phenotype acquire Ag from the surrounding tissues during noninflammatory conditions (35) and migrate to secondary lymphoid organs where an encounter with Ag-specific T cells can result in tolerance to these self-Ags. The amount of self-Ag expressed in the periphery may be one factor that determines whether a naive T cell encounters with APCs results in tolerance or ignorance, as recent studies indicate that high levels of endogenous Ag can lead to the induction of T cell tolerance (36, 37).

Recent approaches to immunotherapy have focused on modifying the maturation status of APCs such that T cell encounters with APCs leads to full activation of naive T lymphocytes. One promising approach has been the engagement of CD40 on the APC via soluble CD40 ligand or agonist anti-CD40 Abs. CD40 on APCs is naturally engaged by the ligand CD154 (CD40 ligand) on activated CD4+ T cells, resulting in APC activation and differentiation (38). CD40 ligation leads to changes in the APC phenotype, including increased surface expression of MHC molecules and costimulatory receptors and the production of high levels of the T cell-activating cytokine IL-12 (39). CD40 ligation results in preservation and expansion of tumor-reactive T cells. In vivo treatment with anti-CD40 Ab resulted in enhanced expansion of epitope I-specific tumor-reactive T cells in vivo.

In this report, the fate of naive T Ag epitope I-specific TCR transgenic (TCR-I) CD8+ T cells was determined after exposure to the endogenous T Ag in 501 T Ag transgenic mice. The results indicate that epitope I-specific CD8+ T cells proliferated and developed an activated phenotype in lymph nodes (LNs) draining areas of T Ag expression; however, these cells failed to acquire effector function. In vivo treatment with anti-CD40 Ab resulted in enhanced expansion of epitope I-specific T cells and promoted the acquisition of effector function in the absence of exogenous immunization. Importantly, this effect was observed even if anti-CD40 treatment was administered after exposure to the epitope I-specific T cells to the endogenous T Ag and led to long-term maintenance of epitope I-specific CD8+ T cells. Thus, in vivo CD40 ligation results in preservation and expansion of tumor-reactive and functional effector T cells that might be targeted for subsequent immunotherapy of tumors.

**Materials and Methods**

**Mice**

Male C57BL/6 (H-2b) mice (4–6-wk-old) were purchased from The Jackson Laboratory (Bar Harbor, ME), maintained in isolator cubicles at the animal facility of the Milton S. Hershey Medical Center. Herpesvirus pseudorabies virus (Ag) and SV40 T Ag were routinely used between the ages of 7 and 12 wk. Line C57BL/6-Tgn (Amy1Tag) 501Kaw, or 501, mice express full-length SV40 T Ag as a transgene from the α-amylase promoter and have been previously described (20, 21). The 501 mice were bred and maintained at the animal research facility of the Milton S. Hershey Medical Center. B6.SJL mice were purchased from Taconic Farms (Germantown, NY) and bred and maintained at the animal research facility of the Milton S. Hershey Medical Center. All animal studies were performed in accordance with guidelines established by the Pennsylvania State University College of Medicine Animal Care and Use Committee under an approved protocol.

**Cloning of the epitope I-specific TCR from CTL clone Y-1**

TCR sequences for both the α- and β-chains were derived from the epitope I-specific CTL clone Y-1 (22). To determine the nucleotide sequence of the combining regions for each subunit, total RNA was extracted from clone Y-1 cells using TriReagent (Sigma-Aldrich, St. Louis, MO) and reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) utilizing an oligo(dT) reverse primer. The TCR α-chain combining region was amplified by PCR utilizing an antisense 3′ constant region oligonucleotide (5′-CGAGATCTTTTAAAAGATAC-3′; Ref. 53) and a 5′ variable region Vα-chain sense oligonucleotide (5′-TCCTCTCACCTTCGGGAAACGCGC-3′; Ref. 53) while the TCR β-chain combining region was amplified using the 5′ antisense constant region oligonucleotide (5′-GTTCGTTATGGTCAAAGTTC-3′; Ref. 53) and a 5′ sense ββ oligonucleotide (5′-AGAAGGCGCGATCGCCCT-3′; Ref. 55). The TCRs and TCRβ PCR products were gel isolated, phosphorylated using T4 polynucleotide kinase (Life Technologies, Grand Island, NY), and rendered blunt-ended with T4 DNA polymerase and subcloned into pUC19. Sequence analysis of the TCR β-chain sequence revealed Jβ1.4 use while analysis of the TCRα sequence revealed Jα1.1 use. Accordingly, 3′ antisense genomic primers corresponding to intron sequences flanking the respective TCRα and TCRβ J regions (Jα1.1, 5′-TGCGGCGCCTTTCGAG-3′ and Jβ1.4, 5′-AGAGATCCCCCCGGCTCTTA-3′) and 5′ sense primers corresponding to 5′ noncoding sequences of Vα3.1 (5′-GGTCCCGGGTTCTCCCAACAAGTCGACCCT-3′; Ref. 53) or Vβ7 (5′-CAACCTTTCGCCAGACCTACGAGGTTAGG-3′; Ref. 55) to amplify the genomic sequences from Y-1 cell-derived DNA and incorporated restriction endonuclease cleavage sites at the ends of each product (α, 5′XmnI, 3′NotI; β, 5′XhoI, 3′KspI/SalI). The amplified genomic V(D)J fragments were subcloned into PUC19 as blunt-ended fragments, the nucleotide sequences were verified, and the fragments were excised by restriction digestion and ligated into the appropriately digested TCR α- or β-expression cassette plasmids (pTrsacs and pTPIcass, respectively; obtained from Dr. D. Mathis, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France; Ref. 57). Recombinant cassette vectors containing the appropriate Y-1 TCR α- and β-chain fragments were identified by restriction digestion analysis, and the presence of the proper V(D)J insert verified by PCR amplification. Plasmids were purified by cesium chloride gradient centrifugation and digested (α, 5′; β, 3′KpnI) to liberate a fragment bearing the respective subunit expression cassettes, which were isolated from agarose gels using the QIAEX II method (Qiagen, Valencia, CA) and eluted directly into microinjection buffer. Fragment solutions were stored at −80°C.

**Generation of T Ag TCR-I mice**

Purified Y-1 TCR α- and β-chain expression cassettes were combined before injection. Microinjection of fertilized embryos from B6D2F1/J mice was performed as previously described (58). The presence of the α- and β-transgene(s) in weanlings was determined at 4 wk of age by PCR analysis of tail-derived DNA using the following primer pairs: Vα5.1–5′-GCCTGTATCTCCTGGCTCCG-3′ and 3′-AAGAAGCGGGAGCATTTCT-3′; Jα1.1–5′-AAGGAAGGCGGGCGGTTCT-3′; Ref. 53) or Vβ5–5′-AGAGAGTTCCCCCGCGGCTCTTA-3′ and 3′-TCCTTCCACCTGCGGAAAGCC-3′/H9252 (Ref. 53) while the TCR Vβ-chain combining region was amplified using the 5′ antisense constant region oligonucleotide (5′-CTTGGGTGGAGTCACATTTCT-3′; Ref. 54) and a 5′ sense ββ oligonucleotide (5′-AGAAGGCGCGATCGCCCT-3′; Ref. 55). The TCRα and TCRβ PCR products were gel isolated, phosphorylated using T4 polynucleotide kinase (Life Technologies, Grand Island, NY), and rendered blunt-ended with T4 DNA polymerase and subcloned into pUC19. Sequence analysis of the TCR β-chain sequence revealed Jβ1.4 use while analysis of the TCRα sequence revealed Jα1.1 use. Accordingly, 3′ antisense genomic primers corresponding to intron sequences flanking the respective TCRα and TCRβ J regions (Jα1.1, 5′-TGCGGCGCCTTTCGAG-3′ and Jβ1.4, 5′-AGAGATCCCCCCGGCTCTTA-3′) and 5′ sense primers corresponding to 5′ noncoding sequences of Vα3.1 (5′-GGTCCCGGGTTCTCCCAACAAGTCGACCCT-3′; Ref. 53) or Vβ7 (5′-CAACCTTTCGCCAGACCTACGAGGTTAGG-3′; Ref. 55) to amplify the genomic sequences from Y-1 cell-derived DNA and incorporated restriction endonuclease cleavage sites at the ends of each product (α, 5′XmnI, 3′NotI; β, 5′XhoI, 3′KspI/SalI). The amplified genomic V(D)J fragments were subcloned into PUC19 as blunt-ended fragments, the nucleotide sequences were verified, and the fragments were excised by restriction digestion and ligated into the appropriately digested TCR α- or β-expression cassette plasmids (pTrsacs and pTPIcass, respectively; obtained from Dr. D. Mathis, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France; Ref. 57). Recombinant cassette vectors containing the appropriate Y-1 TCR α- and β-chain fragments were identified by restriction digestion analysis, and the presence of the proper V(D)J inserts verified by PCR amplification. Plasmids were purified by cesium chloride gradient centrifugation and digested (α, 5′; β, 3′KpnI) to liberate a fragment bearing the respective subunit expression cassettes, which were isolated from agarose gels using the QIAEX II method (Qiagen, Valencia, CA) and eluted directly into microinjection buffer. Fragment solutions were stored at −80°C.
DNA was diagnostic for the presence of the transgenes. Amplification of a 450-bp segment of the p53 gene using previously described primers served as a control for the integrity of the DNA (4, 59). Expression of the transgene product was confirmed by staining lymphocytes from various lymphoid tissues with TCRβ-specific mAb (BD Pharmingen, San Diego, CA) and anti-TCRVo3.1 antisera (generously provided by Drs. B. C. Sim and N. Gascoigne, The Scripps Research Institute, La Jolla, CA) (60) and Db/epitope I tetramer (61). Two founder lines were derived and designated as lines 416 and 422. Line 416 mice were maintained by backcrossing transgene-positive males with C57BL/6 females followed by screening for the presence of the transgene by PCR analysis as described below. Line 416 mice have been backcrossed to C57BL/6 mice for over 10 generations.

Cell lines and media

B6K-Tag1 cells were derived by transformation of C57BL/6 primary kidney cells with plasmid pLMS06-G(DC-1), which encodes a TAg variant containing alanine substitutions of residues N227, F408, and N493. Plasmid pLMS06-G(DC-1) was constructed by the Altered Sites oligonucleotide-directed mutagenesis procedure (Promega) using as a template ssDNA derived from pSelect-ESV (14, 61). Because residues N227, F408, and N493 constitute critical anchor residues within epitopes III, IV, and V, respectively, alanine substitution effectively abrogates both the immunogenicity and antigenicity of epitopes II, III, IV, and V (data not shown).

Cell lines were maintained in DMEM supplemented with 100 U penicillin/ml, 100 µg streptomycin/ml, 100 µg kanamycin/ml, 10 mM HEPES buffer, 10 mM HEPES buffer, 0.075% (w/v) NaHCO3, and 5% FBS. All lymphocyte manipulations were prepared using complete RPMI 1640 medium, which contained 100 U penicillin/ml, 100 µg streptomycin/ml, 2 mM L-glutamine, 10 mM HEPES buffer, 50 µM 2-ME, and 5% FBS. All lymphocyte manipulations were prepared using complete RPMI 1640 medium, which contained 100 U penicillin/ml, 100 µg streptomycin/ml, 2 mM L-glutamine, 10 mM HEPES buffer, 50 µM 2-ME, and 5% FBS.

Synthetic peptides and viruses

Peptides were synthesized at the Macromolecular Core Facility of the Milton S. Hershey Medical Center using an automated peptide synthesizer (9050 MilliGen PepSynthesizer; Millipore, Bedford, MA). Peptides were solubilized in DMSO and diluted to the appropriate concentration with RPMI 1640 medium. Peptides used in these experiments correspond to SV40 T Ag epitopes I (SAINNYAQKL) and influenza virus (Flu) nucleoprotein (NP) 366–374 (ASNENMETM). The recombinant vaccinia virus (rVV) encoding T Ag epitope I as a minigene, rVV-I, virus (Flu) nucleoprotein (NP) 366–374 (ASNENMETM). The recombinant vaccinia virus (rVV) encoding T Ag epitope I as a minigene, rVV-I, has been previously described (28).

Flow cytometric analysis of lymphocyte populations by surface and intracellular cytokine staining

Ex vivo staining of lymphocytes was performed on single-cell suspensions obtained from cervical LNs and spleens, as previously described (61), that were depleted of RBCs by centrifugation over a Ficoll-Paque (Amer sham Biosciences, Piscataway, NJ) gradient according to the manufacturer’s specifications. Production and characterization of the H-2-Db/T Ag system (14, 61). That EBV-generated LCMV-specific CD8+ T cells were injected i.p. with 3 × 105 B6K-Tag1 cells or with 1 × 107 PFU of the indicated rVV. TCR-I mice. Recipients were injected i.v. in the tail vein with lymphocytes derived from line 416 mice containing 5 × 106 clonotypic TCR-I C57Bl/6 T cells, as determined by staining with Db/I tetramer I (DIII Tet) or a control tetramer constructed using the unrelated Dd-restricted epitope NP 366–374 from influenza virus (Flu/Tet). The upper right quadrant represents epitope I-specific CD8+ T cells. The percentage of CD8+ T cells specific for epitope I is indicated.

In vivo proliferation assay

For in vivo proliferation assays (63, 64), RBC-depleted spleen cells from line 416 mice were resuspended at 1 × 107/ml in PBS/0.1% BSA and

FIGURE 1. Line 501 mice are tolerant to TAg epitope I. Six-month-old C57Bl/6 or 501 mice were vaccinated with 1 × 107 PFU rVV-I. Nine days later, splenocytes were harvested and lymphocytes stained with anti-CD8a and either Db/I tetramer I (DIII Tet) or a control tetramer constructed using the unrelated Dd-restricted epitope NP 366–374 from influenza virus (Flu/Tet). The upper right quadrant represents epitope I-specific CD8+ T cells. The percentage of CD8+ T cells specific for epitope I is indicated.

Adoptive transfers, anti-CD40 treatment, and immunizations

Six-month-old C57Bl/6 and 501 mice received adoptive transfers with RBC-depleted lymphocytes derived from spleens and LNs of line 416 mice.
labeled with 5 μM 5- and 6-CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C. Cells were then washed three times in PBS, resuspended in HBSS, and injected i.v at a dose of 5 × 10⁶ clonotypic TCR-I T cells per mouse. After 8 days, spleens and LNs from recipient mice were harvested, and the intensity of CFSE staining was measured among CD8⁺ Db/I Tet⁺ cells.

In vivo cytotoxicity assay

Six-month-old 501 and C57BL/6 mice were injected with 5 × 10⁶ clonotypic TCR-I T cells. After 14 days, RBC-depleted spleen cells from B6SJL congenic mice were pulsed with either 1 μM T Ag epitope I peptide or the control Flu NP 366–374 peptide for 90 min at 37°C, 5% CO₂. Excess peptide was removed with five washes and cells were resuspended at 1 × 10⁶ cells/ml in PBS/0.1% BSA. Epitope I-pulsed cells were labeled with 5 μM CFSE (CFSElow cells) and Flu NP peptide-pulsed cells were labeled with 0.5 μM CSFE (CSFEmid cells) for 10 min at 37°C. Labeling reactions were terminated by the addition of ice-cold PBS followed by two washes in PBS. Mice received an i.v. injection containing a mixture of 2.5 × 10⁶ CFSElow cells and 2.5 × 10⁶ CSFEmid cells in 200 μl HBSS. After 6 h, spleens from recipient mice were harvested, and CFSElow CD45.1⁺ and CFSEmid CD45.1⁺ T cells were quantitated by flow cytometry.

Results

Endogenous T Ag epitope I-specific CD8⁺ T cells are undetectable in 6-month-old 501 mice

We have shown previously that 3-month-old 501 mice immunized with a rVV-encoding epitope I as a minigene induces CTL specific for T Ag epitope I, whereas 6-month-old 501 mice failed to develop detectable levels of epitope I-specific CTL, even after in vitro expansion (19). Because detection of epitope I-specific CTL required both expansion and the development of effector function, it remained to be determined whether the endogenous cells were deleted, failed to proliferate in response to immunization against epitope I, or proliferated but failed to acquire effector function. To examine whether a nonfunctional population of epitope I-specific CD8⁺ T cells accumulated in 6-month-old 501 mice following immunization against epitope I, MHC class I tetramers (Db/I Tet) were utilized to detect the presence of epitope I-specific CD8⁺ T cells directly ex vivo. Nine days after vaccination with rVV-I, C57BL/6 mice developed epitope I-specific CD8⁺ T cells (1.5% of all CD8⁺ cells) detectable by ex vivo staining with Db/I Tet, while epitope I-specific CD8⁺ T cells were undetectable in 501 mice (Fig. 1). Thus, 501 mice failed to accumulate detectable numbers of endogenous epitope I-specific CD8⁺ T cells following specific immunization.

TCR-I T cells accumulate in cervical LNs but fail to acquire effector function following transfer into 501 mice

To develop a system in which the fate of naive epitope I-specific CD8⁺ T cells could be monitored in 501 mice, a mouse line expressing the TCR α- and β-chains of the T Ag epitope I-specific dot plot. B. A dose of 5 × 10⁶ clonotypic TCR-I cells were transferred into 6-month-old 501 or C57BL/6 mice. Seven days after transfer, cells were isolated from cervical LNs. Triple staining was performed using Db/I tetramer, anti-CD8a, and Abs to either CD44 or CD69. Pairs of histograms (upper and lower) represent CD8⁺ T cells that either were (CD8⁺, Db/I Tet⁺) or were not (CD8⁺, Db/I Tet⁻) specific for T Ag epitope I. The percentage of CD8⁺ T cells within each population that had up-regulated CD44 or CD69 is indicated above the marker. C. A dose of 5 × 10⁶ clonotypic TCR-I cells from line 416 mice were transferred into 501 or C57BL/6 mice. At 7 and 14 days after transfer, lymphocytes isolated from the draining cervical LNs and spleen were stimulated with 1 μM of either epitope I peptide or the control peptide NP 366–374 from influenza virus for 6 h in the presence of brefeldin A. CD8⁺ T cells were then stained for the production of intracellular IFN-γ and quantitated by flow cytometry. The percentage of CD8⁺ T cells producing IFN-γ is indicated in the upper right of each quadrant. The data shown are representative of three mice.

FIGURE 3. Activation and expansion of TCR-I T cells in 501 mice in the absence of effector function. A, Five million clonotypic TCR-I cells from line 416 TCR transgenic mice were transferred into 6-month-old 501 or C57BL/6 mice. At 7 and 14 days after transfer, T cells isolated from the spleen and cervical LNs were stained with anti-CD8a and either a T Ag epitope I MHC tetramer (Db/I Tet) or a control tetramer (Db/Flu Tet). Results were quantitated by flow cytometric analysis. The data are representative of three mice per group and the experiment was repeated twice with similar results. The percentage of CD8⁺ T cells that stained positive with each tetramer is indicated in the upper right quadrant of each lymph node. B, Activation and expansion of TCR-I T cells in 501 mice in the absence of effector function. The percentage of CD8⁺ T cells that stained positive with each tetramer is indicated in the upper right quadrant of each histogram.
CTL clone Y-1 was derived. (Fig. 2A). Two founder lines were identified and designated lines 416 and 422. Characterization of line 416 is described in this report. Expression of the transgene products in each line was detected by immunofluorescence staining with Db/I Tet, and Abs specific for the transgenic TCR Vα3.1 and Vβ7 chains. Analysis of spleen cells from line 416 mice revealed that ~94% of the CD8<sup>+</sup> T cells were specific for T Ag epitope I (Fig. 2B), although 100% of the CD8<sup>+</sup> T cells expressed TCRVβ7 (Fig. 2B). This discrepancy was explained by the finding that ~87% of the TCRVβ7<sup>+</sup> CD8<sup>+</sup> T cells coexpressed the transgenic TCRVα3.1 chain (Fig. 2B, left histogram), consistent with the percentage of CD8<sup>+</sup> T cells that stained positive with the Db/I Tet. The specificity of the rabbit anti-Vα3.1 sera is indicated by the lack of positive staining on CD8<sup>+</sup> T cells (data not shown).

The fate of naive TCR-I T cells exposed to endogenous T Ag was monitored following transfer of line 416 spleen cells into 6-mo-old 501 or C57BL/6 mice. Seven and fourteen days after adoptive transfer, spleen and cervical LNs were harvested from recipient mice and the number of epitope I-specific CD8<sup>+</sup> T cells was quantitated by staining with Db/I Tet (Fig. 3A). As a negative control, parallel cell samples were stained with the control tetramer, Db/Flu. In C57BL/6 recipients, ~1.6% of the CD8<sup>+</sup> T cells in the spleen and LNs were specific for epitope I as indicated by staining with Db/I Tet. By contrast, epitope I-specific T cells represented ~8% of the CD8<sup>+</sup> cells in the LNs draining the salivary glands in 501 mice. The frequency of epitope I-specific CD8<sup>+</sup> T cells detected in the spleens of 501 and C57BL/6 mice were similar at day 7. The frequencies of TCR-I cells detected in the cervical LN and spleen remained relatively stable from days 7 to 14 after adoptive transfer into both 501 and C57BL/6 mice. Thus, TCR-I cells accumulate predominantly in the cervical LNs draining this site of T Ag expression in 501 mice.

To determine whether the accumulating TCR-I T cells remained ignorant of endogenous T Ag or had encountered T Ag in the periphery of 501 mice, re-isolated TCR-I T cells were stained for surface expression of CD44 and CD69. These markers are upregulated on CD8<sup>+</sup> T cells following Ag encounter. Of the epitope I-specific CD8<sup>+</sup> T cells (CD8<sup>+</sup>, D<sup>b</sup>/I Tet<sup>+</sup>) that accumulated in the cervical LNs in 501 mice, 78.5% expressed increased levels of CD44 and 54.4% expressed CD69 (Fig. 3B). In contrast, only 20% and 10% of the Db/epitope I negative-staining CD8<sup>+</sup> T cells (CD8<sup>+</sup>, D<sup>b</sup>/I Tet<sup>+</sup>) expressed CD44 and CD69, respectively. TCR-I cells recovered from C57BL/6 mice maintained a naive phenotype as revealed by low surface expression of both CD44 and CD69. Thus, the TCR-I cells that accumulated in LNs draining areas of T Ag expression in 501 mice displayed a phenotype consistent with Ag encounter.

To determine whether Ag encounter by TCR-I T cells in 501 mice resulted in the acquisition of effector function, CD8<sup>+</sup> T cells re-isolated from 501 mice were tested for their ability to produce IFN-γ in response to epitope I. Freshly isolated cervical LN or spleen cells were stimulated in vitro with epitope I peptide or a control peptide (Flu NP 366–374) in the presence of brefeldin A for 6 h and then stained for accumulation of intracellular IFN-γ. As expected, cells re-isolated from C57BL/6 mice that were never exposed to T Ag failed to produce IFN-γ in response to epitope I peptide (Fig. 3C). Lymphocytes isolated from 501 mice, which developed an Ag experienced phenotype in LNs draining sites of T Ag expression (Fig. 3B), also failed to produce significant amounts of IFN-γ (Fig. 3C). Although 8–9% of CD8<sup>+</sup> T cells from the cervical LNs of 501 mice were positive by tetramer staining (Fig. 3A), <1% of the CD8<sup>+</sup> T cells from these same LNs were capable of producing IFN-γ after stimulation with epitope I peptide. Thus, although TCR-I T cells accumulated in LNs draining the site of T Ag expression and developed an activated phenotype in 501 mice, they failed to acquire effector function.

**TCR-I T cells exposed to endogenous T Ag respond inefficiently to subsequent immunization.**

To determine whether naive TCR-I T cells exposed to the endogenous T Ag in 501 mice are compromised in their ability to respond to T Ag epitope I in vivo, 6-mo-old 501 and control C57BL/6 mice were given adoptive transfers with 5 × 10<sup>6</sup> clonotypic TCR-I T cells, rested for 2 wk and then immunized with rVV-I. Representative mice from each group were sacrificed at day 14, before immunization, to determine the frequency of TCR-I T

---

**FIGURE 4.** Naive TCR-I T cells become tolerant following transfer into 501 mice. Six-month-old 501 and C57BL/6 (B6) mice were reconstituted with 5 × 10<sup>6</sup> clonotypic TCR-I T cells and rested until day 14 following adoptive transfer. Lymphocytes from spleen and LN of representative mice were analyzed at day 14 to determine the percentage of CD8<sup>+</sup> T cells specific for T Ag epitope I by staining with MHC tetramers (mouse #1 and #2). Half of the remaining mice in each group were immunized with 1 × 10<sup>7</sup> PFU rVV-ES-I at day 16 postadoptive transfer and then sacrificed 9 days later (day 25 post-TCR-I transfer) for analysis of spleen and LN populations. The percentage of CD8<sup>+</sup> T cells that stained positive with Db/I Tet is indicated in upper right of each histogram for both immunized (mouse #3 and #4) and unimmunized (mouse #5 and #6) mice. The data represent 100,000 events. *, Only 10,000 events were collected for B6#3 LN.
FIGURE 5. Treatment of 501 mice with anti-CD40 Ab enhances proliferation and induces effector function in TCR-I T cells. A, Five million clonotypic TCR-I cells were transferred into 6-mo-old 501 mice that also received 100 μg of purified FGK45 or rat IgG on the day before and the day after cell transfer. Seven days after transfer, lymphocytes were isolated from the spleen and cervical LNs and CD8^+ T cells were stained with Db/epitope I tetramer (Db/I Tet) or stained for the production of IFN-γ following 6 h of in vitro stimulation with epitope I peptide. The number of epitope I-specific CD8^+ T cells was quantitated by FACS analysis. The percentage of CD8^+ T cells specific for epitope I is indicated in the upper right quadrant. (Figure legend continues)
cells present at this time point. TCR-I T cells were present at a frequency of 1–2% of CD8+ T cells in both the spleen and cervical LNs of C57BL/6 recipients (Fig. 4B). As shown previously (Fig. 3), while the frequency of TCR-I T cells present in the spleen of 501 mice at day 14 posttransfer was similar to that detected in C57BL/6 mice, increased numbers of TCR-I T cells accumulated in the cervical LNs of 501 mice at this time point (Fig. 4A).

Immunization of C57BL/6 mice with rVV-I resulted in a dramatic expansion of TCR-I T cells (~10-fold) in both the spleen and LNs by day 9 postimmunization (Fig. 4B). It should be noted that the endogenous epitope I-specific CD8+ T cell response in C57BL/6 mice represents ~1.5% of the CD8+ T cells by 9 days postimmunization with rVV-I (see Fig. 1). In contrast, TCR-I T cells expanded ~2–3-fold in the spleens of 501 mice with no apparent increase in the frequency of TCR-I T cells in the cervical LNs (Fig. 4A). Low levels of TCR-I T cells were detected in unimmunized 501 and C57BL/6 mice at this same time point, although the percentage of TCR-I T cells detected in 501 mice had apparently decreased by 25 days postadoptive transfer. These results indicate that exposure of naive TCR-I T cells to the endogenous T Ag in 501 mice compromises their ability to respond to subsequent immunization, consistent with the development of tolerance to T Ag epitope I.

**Expansion of effector TCR-I T cells following conditioning of 501 mice with activating anti-CD40 Ab**

Professional APCs can be stimulated to increase their Ag-presenting activity and immunogenic potential by ligation of their CD40 receptor (39, 45, 65). This is mediated through engagement of CD154, a TNF family member expressed on activated CD4+ T cells, platelets, and mast cells (38). The engagement of CD40 by CD154 can be replaced by the administration of the FGK45 agonistic Ab to CD40, bypassing the requirement for CD4+ T cell help in the activation of naive CD8+ T cells (43, 44). Thus, the ability of FGK45 administration to promote the acquisition of effector T cell function among adoptively transferred TCR-I T cells following transfer into 501 mice was determined.

TCR-I cells were transferred into 501 mice treated with FGK45 or an isotype-matched control Ab. After 7 days, TCR-I cells isolated from cervical LNs and spleens were quantitated by staining with Db/I Tet and epitope I peptide-induced IFN-γ production. Line 501 mice treated with FGK45 accumulated 2- and 3-fold more TCR-I cells in cervical LNs and spleens, respectively, compared with mice that received control Ab (Fig. 5A). Importantly, treatment with FGK45 resulted in an increase in the fraction of TCR-I T cells that produced IFN-γ in response to epitope I peptide. The ratio of IFN-γ-producing cells to Db/Ipeptide I tetramer-positive cells in the LNs was 0.6 in FGK45-treated mice compared with 0.18 in control Ab-treated mice. Thus, treatment with FGK45 led to enhanced accumulation of TCR-I T cells in 501 mice that displayed increased effector function.

We next determined whether the increased accumulation of TCR-I T cells within the lymphoid organs of FGK45-treated 501 mice could be explained by increased proliferation of TCR-I T cells. Line 416 spleen cells were labeled with CFSE before transfer into 501 or C57BL/6 mice. CFSE is a stable fluorescent dye that decreases in intensity by approximately one-half with each cell division, due to equal partitioning into daughter cells, and can therefore be used to measure the rate and extent of cell proliferation. Over 70% of the CD8+ Db/I Tet+ T cells recovered from the cervical LNs and spleen of 501 mice that received FGK45 had lost the CFSE label by 8 days posttransfer, indicating that the majority of accumulating cells had undergone more than seven divisions (Fig. 5B). In contrast, only a small fraction (3%) of TCR-I cells isolated from the cervical LNs of 501 mice given control Ab had divided enough times to lose the CFSE stain, with most cells having undergone three to five cell divisions. Similarly, only 22% of the TCR-I T cells re-isolated from the spleen of control Ab-treated 501 mice had undergone more than seven divisions and lost CFSE fluorescence. Thus, in vivo ligation of CD40 resulted in an increase in the rate of TCR-I T cell proliferation (Fig. 5B) as well as an increase in the total number of TCR-I T cells that accumulated in the secondary lymphoid organs (Fig. 5A). TCR-I cells transferred into C57BL/6 mice only proliferated if the mice were challenged with rVV-I, which resulted in the proliferation of all TCR-I T cells more than seven cell divisions and a corresponding loss of the CFSE label (Fig. 5B).

Although these results indicated that FGK45 treatment could promote the acquisition of T cell effector function if administered before the transfer of TCR-I T cells into 501 mice, a more realistic scenario was whether FGK45 treatment could promote the accumulation of effector T cells that had been previously exposed to the tolerogenic environment of 501 mice. Thus, the effect of administering FGK45 Ab 7 days posttransfer of TCR-I T cells was determined. This corresponds to a time point when TCR-I T cells had expanded in 501 mice, but failed to develop effector function (Fig. 3A). This approach resulted in the accumulation of similar numbers of TCR-I T cells in the draining LNs by day 14 postadoptive transfer whether or not FGK45 was administered before transfer of TCR-I T cells or at 7 days posttransfer (Fig. 5C). Tetramer analysis, compare day −1, +1 to day 7, 8). By comparison, significantly more TCR-I T cells accumulated in the spleens of 501 mice if FGK45 was administered before cell transfer (62.4 vs 26.4% of CD8+ T cells). This difference was less apparent by day 21 posttransfer. The ratio of IFN-γ-producing cells to Db/I+ CD8+ T cells also was reduced at day 14 in mice that received delayed treatment with FGK45 (LN, 0.31; spleen, 0.51) compared with mice pretreated with FGK45 (LN, 0.54; spleen, 0.96). This discrepancy was not apparent at day 21, perhaps reflective of the stable memory T cell pool. These results indicate that FGK45 treatment remained effective in promoting the acquisition of T cell function if administered after exposure of naive TCR-I T cells to the tolerogenic environment of 501 mice and established a stable memory T cell population capable of producing IFN-γ in response to Ag.

Some FGK45-treated 501 mice that received TCR-I T cells at 6 mo of age were monitored for the long-term maintenance of...
A classic function of a CD8 effector function TCR-I T cells from anti-CD40-treated 501 mice have in vivo associated with the long-term survival of TCR-I T cells in 501 mice. Thus, FGK45 treatment is associated with the long-term survival of TCR-I T cells in 501 mice.

**FIGURE 6.** TCR-I T cells transferred into 501 mice treated with anti-CD40 Ab are cytotoxic in vivo. TCR-I T cells were transferred into 6-month-old 501 (A) or C57BL/6 (B) mice that received 100 μg FGK45 or an isotype-matched control Ab the day before and the day after cell transfer. An additional group of C57BL/6 mice received TCR-I T cells and subsequent immunization with B6/K-TagI cells that express a T Ag variant in which epitopes II/III, IV, and V are inactivated. After 14 days, mice were injected i.v. with a mixture of 2.5 × 10⁶ T Ag epitope I peptide-pulsed B6/SJL (CD45.1⁺) spleen cells labeled with 5 μM CFSE and 2.5 × 10⁶ Flu NP 366–374 peptide-pulsed B6/SJL spleen cells labeled with 0.5 μM CFSE. After 6 h, CD45.1⁺ cells derived from recipient spleens were examined by FACS analysis to quantitate the number of CFSE²⁺ and CFSE⁺ cells that were recovered.

**Discussion**

Recent studies have indicated that the loss of CD8⁺ T cell responsiveness to parenchymal Ags in the periphery is initiated by the cross-presentation of these self-Ags on bone marrow-derived APCs, such as DCs, to naive T cells (8, 34). CD8⁺ T cell recognition of endogenous Ag results in the modulation of surface molecules indicative of T cell activation, followed by T cell proliferation (28, 66). These T cells, however, fail to obtain effector function and are typically deleted within a period of a few weeks (28, 30, 66). This scenario represents a major drawback for immunotherapeutic strategies to cancer that target tumor-associated self-Ags recognized by CD8⁺ T cells. The results presented in this report define a system in which the onset of peripheral CD8⁺ T cell tolerance to an endogenous tumor Ag can be readily observed. Naïve CD8⁺ T cells specific for T Ag epitope I encountered the endogenous T Ag after transfer into 501 mice, resulting in the up-regulation of activation markers and T cell proliferation, but failed to acquire effector function. That the TCR-I T cells became functionally compromised and did not simply maintain a naïve phenotype was evidenced by their lack of responsiveness to subsequent immunization against epitope I. In addition, TCR-I T cells disappeared from the peripheral lymphoid organs of 501 but not C57BL/6 mice around 4 wk postadoptive transfer, consistent with a deletional mechanism of tolerance induction.

Several approaches have been proposed to block or reverse the onset of peripheral T cell tolerance for the purpose of salvaging potentially therapeutic T cells within the host T cell repertoire. Because the role of the APC is central for complete activation of naïve CD8⁺ T cells, triggering APC function via ligation of the CD40 receptor has received recent attention (67). This approach was shown to block the onset of peripheral T cell tolerance in vivo if agonistic anti-CD40 Ab was administered along with specific immunization (46–48, 68). The ability of anti-CD40 ligation to lead to the priming of CD8⁺ T cells against endogenous self-Ags or preexisting tumors, however, has yielded a different set of results. Kedl et al. (50) demonstrated that the administration of anti-CD40 Ab into tumor-bearing mice in the absence of specific immunization led to the enhanced deletion of tumor-specific CD8⁺ T cells. More recently, Hernandez et al. (69) found that administration of anti-CD40 enhanced the proliferation of TCR transgenic CD8⁺ T cells specific for an influenza virus hemagglutinin (HA) epitope in HA transgenic mice, but failed to result in the induction of effector function. In contrast to these studies, we demonstrate that administration of FGK45 into 501 mice resulted in increased
proliferation of TCR-I T cells within the secondary lymphoid organs as well as the acquisition of effector functions, including the ability to destroy Ag epitope I-expressing target cells in vivo. Although the basis for this difference is not readily apparent, some possible explanations may include differences in the antigenic systems utilized, different levels of endogenous Ag expressed, or differences in the route of delivery or amount of anti-CD40 Ab utilized. Hernandez et al. (69) demonstrated that CD4+ T cell effector function could be induced in HA-specific CD8+ T cells if activated HA-specific CD4+ T cells were coinjected or if IL-12 was coadministered with the anti-CD40 Ab. These results suggested that additional signals, such as cytokines, were required for the full activation and subsequent acquisition of effector functions by naive HA-specific TCR transgenic CD8+ T cells in HA transgenic mice. One possible explanation for the success of CD40 ligation alone to lead to the acquisition of effector function among naive TCR-I T cells is that the CD4+ T cell compartment might not be tolerant in 501 mice, in contrast to the HA transgenic mice, such that additional signals could be provided by the endogenous CD4+ T cells in 501 mice to either the APCs or the TCR-I T cells. Preliminary results in 501 mice, however, indicate that the presence of CD4+ T cells is not required for anti-CD40 administration to promote the acquisition of effector function by TCR-I T cells (data not shown). An alternative explanation is that the TCR-I T cells might not require additional signals other than those delivered by CD40-induced APCs. Future studies will assess the phenotype of CD40-induced APCs from 501 mice to determine whether these APCs alone are fully capable of activating naive TCR-I T cells.

Differences in the level of Ag expression or the antigenic properties of the Ag systems investigated might also explain the observed differences. Previous investigations assessing the ability of H-2-Kk-restricted, T Ag epitope specific TCR transgenic CD8+ T cells to respond to endogenous T Ag expressed from the insulin promoter demonstrated that transgenic expression of the full-length T Ag (70), but not a nontransforming truncated protein (26), led to the induction of autoreactivity. These results suggested that the oncogenic process initiated by full-length T Ag might increase the priming of autoreactive T cells, while expression of a nontransforming mutant T Ag resulted in immunologic ignorance (26). Thus, the full-length T Ag might be capable of providing additional signals to the APCs or other immune effector cells, such that CD40 ligation alone results in full activation of the naive CD8+ T cells in 501 mice.

A few studies have indicated that the administration of anti-CD40 Ab can lead to a more rapid onset of tolerance (50, 71). Our analysis indicates that TCR-I T cells exposed to the endogenous T Ag for one week remained responsive to the activating effects of anti-CD40 administration. Whether these T cells will remain responsive for longer periods of time is unknown, but our data indicate that at least some of the T cells can be rescued by anti-CD40 administration. One potential difference between our study and those that showed anti-CD40-induced deletion of responsive T cells is that the mice in previous studies were given multiple injections of anti-CD40 Ab over the course of the experiment vs two doses of FGK45 at 1-day intervals administered in this study. Mauri et al. (71) assessed the effects of anti-CD40 ligation on the development of collagen-induced arthritis following repeated injections of anti-CD40 into collagen type II-treated animals. These authors found that repeated anti-CD40 administration decreased the onset of disease severity and skewed the T cell response from Th type 1 to type 2. They suggested that anti-CD40 treatment might skew the dominant APC from DCs to B cells, which favor a Th type 2 response rather than a Th type 1 response. Thus, repeated injections of anti-CD40 might be detrimental for maintaining CD8+ T cell responses, suggesting that the timing of anti-CD40 administration needs to be carefully assessed.

One important aspect of the present study is the finding that administration of anti-CD40 led to the establishment of a stable memory population of functional TCR-I T cells on a systemic level. High levels of TCR-I T cells were detected in 501 mice by day 21 postadoptive transfer regardless of whether the mice were treated with anti-CD40 at the time of TCR-I T cell transfer or 1 wk later. In addition, a significant proportion of these T cells maintained the ability to produce IFN-γ upon stimulation in vitro. We also found that these T cells could be detected in the peripheral blood of anti-CD40 treated 501 mice up to 1 year following adoptive transfer, ranging from 3 to 18% of the total CD8+ T cells. This finding is particularly intriguing in the light of recent studies that demonstrated that CD4+ effector T cells and CD8+ memory T cells remained susceptible to the effects of peripheral tolerance to self-Ag in HA transgenic mice (72, 73). We have yet to determine whether the TCR-I long-term memory T cells in 501 mice can effectively control tumor progression in 501 mice or maintain the ability to destroy epitope I-expressing target cells in vivo. These memory T cells, however, retained the ability to produce IFN-γ following in vitro stimulation (data not shown). Thus, effector T cells generated in the presence of anti-CD40 treatment establish a stable memory T cell pool that might later be tapped for immunotherapy of developing tumors.

In conclusion, the results presented in this study provide encouraging support for the use of anti-CD40 treatment to generate significant CD8+ T cell responses to tumor-associated self-Ags. Clearly the conditions for anti-CD40 treatment must be carefully evaluated for each particular Ag system to provide maximal stimulatory responses and prevent accelerated deletion of responding T cells. Key to this approach is the presence of a population of T cells capable of responding to the target Ag, which might consist of the residual endogenous T cell response, or be derived from adoptive transfer. Thus, in cases where T cell precursors are limiting, the use of specific immunization or ex vivo expansion to generate increased numbers of effector T cells in combination with in vivo anti-CD40 treatment might be desirable.

Acknowledgments

We thank Melanie Epler, Andrew Gaydos, and Rebecca Pound for excellent technical assistance.

References

CD40-MEDIATED PRIMING AGAINST ENDOGENOUS TUMOR Ag


