Reduced Functional Avidity Promotes Central and Effector Memory CD4 T Cell Responses to Tumor-Associated Antigens

Stefano Caserta, Joanna Kleczkowska, Anna Mondino and Rose Zamoyska

*J Immunol* 2010; 185:6545-6554; Prepublished online 3 November 2010; doi: 10.4049/jimmunol.1001867

http://www.jimmunol.org/content/185/11/6545
Reduced Functional Avidity Promotes Central and Effector Memory CD4 T Cell Responses to Tumor-Associated Antigens

Stefano Caserta,* Joanna Kleczkowska,* Anna Mondino,† and Rose Zamoyska*

The effect of TCR signals on the differentiation of memory T cells is poorly defined. Conventional wisdom suggests that high-avidity interactions are best for the selection of vaccine Ag candidates or T cell specificities for adoptive T cell therapy to stimulate robust responses. However, in conditions of Ag persistence, high-avidity clones might exhaust and fail to form long-lived protective memory. We have manipulated the functional avidity of CD4 T cells by reducing expression of Lck, a key kinase involved in TCR triggering. Using a mouse model, we followed tetramer-positive T cells responding to a tumor Ag expressed by an adenocarcinoma. We show that reducing the functional avidity increased effector–effector memory responses and improved the generation of self-renewing, recirculating, tumor Ag-specific memory phenotype CD4 T cells. Moreover, such cells together with wild type CD8 T cells were better able to control tumor growth. Mechanistically, reducing Lck prolonged IL-2 production and cell turnover in the central memory population while reducing expression of exhaustion markers in the face of chronic Ag. Our data indicate that, in situations of persistent Ag challenge, generating T cells with reduced functional avidity may elicit more effective immune responses. The Journal of Immunology, 2010, 185: 6545–6554.

The development of effective T cell memory is a hallmark of the adaptive immune response and the goal of vaccination strategies. In recent years, a good deal has been learned about the process that drives naive T cells to become effector and memory populations, and a number of genetic programs relevant to the adoption of an effector or memory fate have been described (1, 2). However, we still have a poor understanding of the signaling events that initiate these decisions of fate, and many questions remain about the relationships between the lineages that give rise to effector and memory subpopulations. In particular, debate has focused on whether there is a progressive differential differentiation path, such as naïve T cell → effector T cell → memory T cell (3), or whether effector or memory cells independently differentiate directly from naïve T cells (e.g., as a consequence of an early asymmetric division) as has been suggested in some studies (4). Clearly the initial encounter of TCRs on naïve T cells with Ag:MHC complexes (signal 1) and the interactions between costimulatory molecules and their ligands on APCs (signal 2), together with the surrounding inflammatory cytokine milieu (signal 3), play an important part in the subsequent differentiation decisions of T cells. However, the biochemical consequences of these interactions and an understanding of how they affect cellular fate are poorly understood.

Efforts to identify signaling parameters downstream of the TCRs that influence the differentiation of effector and memory fates have largely concentrated on manipulating Ag dose or exposure and monitoring subsequent outcomes. CD8 T cells seem to be able to generate memory cells as efficiently to both low and high avidity peptides (5) and following brief Ag exposure (6–8). For CD4 T cells it appears that higher effective Ag doses (9–11) and longer Ag exposure times (12, 13) are required for efficient memory cell generation. It is unclear whether this apparent requirement for stronger TCR signaling to generate CD4 memory T cells represents a fundamental difference in the differentiation potential between CD4 and CD8 T cells or whether it reflects differences in their activation thresholds or functional avidity. The latter is influenced by a variety of factors, such as the affinity of TCR-peptide:MHC binding, the density of the ligand, and the distribution and composition of signaling molecules within the T cell, that dictate its sensitivity to being triggered through the TCR. Alterations in any one or all of these components can affect the differentiation outcome.

In contrast to acute viral and bacterial infections that are cleared rapidly, tumors and persistent pathogens present an additional problem to the development of effector and memory T cells. In conditions of Ag persistence, the generation and maintenance of memory-phenotype CD4 T cells is impaired, and repeated Ag exposure drives terminal differentiation of precursors into short-lived effectors with a failure to accumulate systemic memory-like responses (14, 15). Thus, in contrast to in vitro studies showing that higher degrees of TCR stimulation can favor differentiation to memory (16, 17), conditions of chronic Ag persistence in vivo can result in clonal exhaustion (18). Whether exhaustion results from environmental pressures—such as the nature of the APC, the contribution of TLR signals, or cytokine availability in niches—or whether it is a result of intrinsic differences related to the functional avidity of individual T cell clones is unknown.

*Institute for Immunology and Infection Research, The University of Edinburgh, Edinburgh, United Kingdom; and Cancer Immunotherapy and Gene Therapy Program, Department of Oncology, San Raffaele Scientific Institute, Vita-Salute San Raffaele University, Milan, Italy

Received for publication June 7, 2010. Accepted for publication September 26, 2010. This work was supported by Cancer Research UK Project Grant R40534 and by the Medical Research Council UK. S.C. was initially supported by a fellowship from the International Ph.D. Program in Molecular Medicine (Vita-Salute San Raffaele University, Milan, Italy)

Address correspondence and reprint requests to Rose Zamoyska, The University of Edinburgh, West Main Road, EH9 3JT Edinburgh, United Kingdom. E-mail address: Rose.Zamoyska@ed.ac.uk

Abbreviations used in this paper: BM, bone marrow; DC, dendritic cell; dLN, draining lymph nodes; Dox, doxycyclin; LACK, Leishmania analogue of the receptors of activated C kinase; LN, lymph node; mLN, nondraining lymph nodes; qPCR, quantitative PCR; TCM, T central memory; TE, T effector; Tet, Tetramer; WT, wild type.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10S16/00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1001867
To address the question of whether the functional avidity of T cells can affect the formation and persistence of effector and memory T cells, we used a mouse model in which we can alter expression of Lck, the key proximal kinase involved in triggering the TCR, using a tetracycline-inducible transgene (19). We have shown previously that reducing or removing Lck alters the threshold at which cells can be triggered, thus reducing their functional avidity (20). We examined how this reduction in sensitivity to stimulation affected the response to a persistent tumor by following the response of tetramer+ cells from a TCR-β transgenic mouse to a defined tumor Ag. Surprisingly, we found that lowering the functional avidity favored the generation of recirculating, self-renewing, multipotential, central memory-like CD4 T cells together with a corresponding persistence of effector phenotype cells. In addition, these memory cells appeared to be able to continuously seed the effector pool. In contrast, central memory phenotype cells expressing endogenous Lck showed evidence of exhaustion and had correspondingly reduced effector cell numbers. In addition, lower levels of Lck resulted in CD4 T cells that, with WT CD8 T cells, were better able to control tumor growth. These data show that in situations of chronic Ag persistence, a lowering of functional avidity can promote more effective CD4 T cell responses.

Materials and Methods

Mice and tumor lines

16.2β transgenic mice on a BALB/c (H-2b) background express a transgenic TCR-β-chain (Vb4) specific for the immunodominant peptide (Leishmania analog of the receptors of activated C kinase [LACK], FSP51LEHIPVSVGSDW), derived from the Leishmania major LACK protein and presented in the context of H-2d; MHC class II molecules (21). Lck+ to every 2 d (19) were backcrossed to 16.2β mice to give 16.2βmB+mLck+/− (H-2b, C57BL/6 background) strains that were intercrossed with rTACm/Lck+/− (H-2b, C57BL/6 background) to generate 16.2β Lck-inducible mice (H-2b/−/). 16.2βmB+mLck+/− were intercrossed with C57BL/6 mice to generate 16.2β H-2b control mice. Lck-inducible intercrosses were given doxycycline (Dox) in food (1 mg/g) continuously from gestation. All mice were bred and maintained in a specific pathogen-free facility at the Medical Research Council National Institute for Medical Research under the U.K. Home Office and local guidelines.

TS/A-LACK mouse mammary adenocarcinoma cells (4 × 105) (14) were inoculated s.c. in the right flank. The axillary, brachial, and inguinal lymph nodes (LN) draining the site of tumor injection were resected as to tumor-draining LN (dLN). The brachial, axillary, and inguinal LNs derived from the contralateral flank were referred to as tumor nondraining LNs (ndLN). Tumor growth was monitored every other day by measuring the tumor phenotype cells. In addition, these memory cells appeared to be able to continuously seed the effector pool. In contrast, central memory phenotype cells expressing endogenous Lck showed evidence of exhaustion and had correspondingly reduced effector cell numbers. In addition, lower levels of Lck resulted in CD4 T cells that, with WT CD8 T cells, were better able to control tumor growth. These data show that in situations of chronic Ag persistence, a lowering of functional avidity can promote more effective CD4 T cell responses.

Mouse T cell primary cultures

LN cells were labeled with the fluorescent dye CFSE as described (23). Cells were cultured at 37°C in complete medium (RPMI 1640, 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 2.5 × 10−5 M 2-BE; Invitrogen, Carlsbad, CA) for the number of days specified in each experiment. Dox (2 μg/ml) (Sigma-Aldrich, St. Louis, MO) was added in vitro every 2 d. Anti-CD3 (145-2C11, 0.01 μg/ml) and anti-CD28 (37.51; 5 μg/ml) mAb were coated on plates for 1 h at 37°C in PBS, and the wells were washed twice before adding cells. LACK-specific tetramers plus anti-CD28 Ab were coated onto Latex beads (Invitrogen) as described previously (24); 10(4) CD4+ LN-derived cells were cultured with 2.5 × 10(4) beads.

Flow cytometry

1-Aβ/LACK multimer staining was performed as described (14). Cells were labeled with PE-TexasRed- (CalTag Laboratories, Burlingame, CA), PE-APC-, or PerCP-labeled Abs: anti-CD4, anti-CD44, anti-H-2b, anti-CD62L, anti-CD8α, anti-CD11b, and anti-B220 mAb (BD Pharmingen, San Diego, CA; eBioscience, San Diego, CA). TO-PRO-3 (1 nM final concentration; Molecular Probes, Invitrogen) was added to the sample just before FACS analysis. Events were collected (3 × 107 CD4+ or at least 107 CD4+ 1-Aβ/LACK+) by excluding all the anti-CD11b, anti-B220+, anti-CD8α, and TO-PRO-3+ events. Cells were stained with directly conjugated Abs for 15 min at 4°C, washed, and acquired on FACS Calibur, LSR I, or LSR II flow cytometers (BD Biosciences, San Jose, CA).

Quantitative PCR analyses

Highly purified (>97%) T central memory (TCM) and T effector (TE) were obtained by sorting with MOFLO cell-sorters (Beckman Coulter, Brea, CA). Cell pellets (1 × 107) were lysed for RNA extraction using the RNeasy Mini Kit (Qiagen, Valencia, CA) protocol. RNA was reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative PCR (qPCR) reactions were performed in the presence of TaqMan primers specific for mouse Hprt, KlrG1, Pdcd1 (PD-1), Pdml (Blimp1), and Casp3 (Applied Biosystems, Foster City, CA) and normalized to Hprt expression levels for each sample.

Statistical analysis

Two-tailed Student t test (unpaired) was used to assess statistically significant changes between groups.

Results

Low expression of Lck reduces the functional avidity of T cells

We used an experimental system in which we could follow the response of CD4 T cells to a model tumor-associated Ag. 16.2β mice are transgenic for a rearranged TCR-β-chain isolated from a CD4 T cell clone specific for a Leishmania major Ag, LACK (25). To alter the functional avidity of the responding T cells, mice expressing a tetracycline-inducible Lck transgene (19) were backcrossed to 16.2β mice (16.2β/Lck+/LckKO mice) and subsequently intercrossed with mice expressing the Tet-Ont transactivator, rTA (19). These mice were on an endogenous Lck−/− background, and expression of Lck in the offspring was exclusively induced and maintained by the inclusion of Dox in food (16.2β LckON) or, when required, Lck expression could be extinguished by withdrawal of Dox for at least 7 d (16.2β LckOff). Because the Lck-inducible mice were of MHC H-2a/b haplotype and heterozygous for the TCRβ transgene, control mice were generated by intercrossing 16.2β/Lck+/LckKO+ mice with C57BL/6 mice. Although heterozygous for endogenous Lck, these controls behaved similarly to homozygous 16.2β animals (data not shown) and are hereafter referred to as WT 16.2β mice.

In the context of a polyclonal TCR-α repertoire, LACK-specific T cells occur at a frequency of ~0.5% in unchallenged 16.2β control animals, which is higher than that found in naive BALB/c animals and can be followed by staining with specific peptide: MHC multimers (Fig. 1A). The LACK tetramer was shown previously to have essentially no reactivity with irrelevant T cells, because staining was undetectable on DO11.10 TCR transgenic cells (24). The frequency of LACK+ clonotypic T cells in 16.2β LckON mice was comparable to WT controls, and they had a naive, CD44low phenotype (Fig. 1A). As described previously (19, 25), the amount of Lck in naive T cells from LckON mice is ~10–20% of WT levels and is found primarily in association with CD4 (20). Lck prevents CD4 internalization into endosomes and thus contributes to the maintenance of CD4 surface expression (26). Consequently, CD4 surface expression was lower in LckON cells (Fig. 1A) and acted as a convenient indicator of Lck levels in

6546 REDUCING AVIDITY IMPAIRS MEMORY CELL EXHAUSTION

Downloaded from http://www.jimmunol.org/ by guest on November 18, 2017
these cells (20). The proportions of naive and memory cells in nonimmunized 16.2β LckON mice were similar to those found in WT 16.2β mice (Fig. 1A, right panel). Numbers in the quadrants of 16.2βWT and LckON mice are the mean frequency ± SD from 10 animals. LckON LN cells cultured with a titration of anti-CD3 plus 5 μg/ml anti-CD28 require more stimulation to achieve equivalent CD69 upregulation to WT cells. C. Proliferation of LckON CFSE-labeled cells requires CD28 costimulation and is enhanced by supplementation of Dox every 2 d to cultures in vitro. D. LN cells from LckON mice proliferate to tetramer plus αCD28-coated beads only in the presence of Dox and show less cell death (TOPRO-3 staining) after stimulation for 7 d. Data are representative of three independent experiments.

**FIGURE 1.** LckON naive CD4 T cells have a higher stimulation threshold in vitro. A, Staining of LN cells ex vivo shows few I-Aβ/LACK tetramer+ (Tet+) T cells in BALB/c mice, whereas similar frequencies of Tet+ cells are present in 16.2βWT and LckON mice (top panel) and they have a naive CD44lo phenotype (bottom panel). B, LckON LN cells cultured with a titration of anti-CD3 plus 5 μg/ml anti-CD28 require more stimulation to achieve equivalent CD69 upregulation to WT cells. C, Proliferation of LckON CFSE-labeled cells requires CD28 costimulation and is enhanced by supplementation of Dox every 2 d to cultures in vitro. D, LN cells from LckON mice proliferate to tetramer plus αCD28-coated beads only in the presence of Dox and show less cell death (TOPRO-3 staining) after stimulation for 7 d. Data are representative of three independent experiments.

The Journal of Immunology 6547
by ~20-fold and were more highly represented near the site of tumor growth (i.e., the tumor-draining LN, dLN; Fig. 2A). In contrast to the naive phenotype, CD44hiCD62Llo, of control cells from nonimmunized mice (Fig. 3A), the day 12 responding cells were largely of a TE-effector memory, CD44hiCD62Llo, phenotype (hereafter collectively referred to as TE; Figs. 2B, 3B, upper panel) and were also detected to a lesser extent in a distal lymphoid site (usually the contralateral flank from the localized site of tumor development, ndLN). In addition to a TE population, d12 tumor-bearing WT mice had a distinct population of CD44hiCD62Lhi, phenotype (hereafter collectively referred to as TCM; Figs. 2B, 3B, upper panel) and were also detected to a lesser extent in a distal lymphoid site (usually the contralateral flank from the localized site of tumor development, ndLN). In addition to a TE population, d12 tumor-bearing WT mice had a distinct population of CD44hiCD62Lhi cells that were distributed approximately equally between the dLN and ndLN (Figs. 2C, 3B, upper panel). The ability to redistribute systemically around lymphoid organs is one of the hallmarks of central memory T cells (14, 27), and this together with the phenotype suggested that these CD44hiCD62Lhi cells were a TCM population. By day 19, when the tumor load had increased substantially, there was an ~5-fold drop in the total number of LACK+CD44hi cells in the dLN compared with that found at day 12, whereas the ndLN numbers of LACK+CD44hi cells reverted to that found in naive animals (Fig. 2A). The remaining LACK-specific cells at day 19 were phenotypically CD44hiCD62Llo effector cells mainly confined to the draining LN (Figs. 2B, 3B, lower panel), and there was almost a complete absence of TCM cells in either dLN or ndLN, both in numbers (Fig 2C) and as a percentage (Fig. 3B, lower panel), consistent with exhaustion of the tumor specific memory response.

Challence of 16.2b LckON mice with tumor resulted in a comparable LACK-specific response at day 12 when tumor burden was low. Similar to WT 16.2b mice, there was an increase in LACK+CD44hi cells in both the dLN and ndLN at this time (Fig. 2A) with TE and TCM detectable at both sites (Fig. 3C, upper panel). Remarkably, by day 19 this situation altered considerably and, in contrast to WT mice, we observed sustained accumulation of LACK-specific CD44hi cells in both dLN and ndLN of LckON mice (Figs. 2A, 3C, lower panel) regardless of the increased tumor load, which was a similar volume in both types of mice (data not shown). Moreover, both TE and TCM phenotype cells accumulated (Fig. 3C, lower panel). Both TE (Fig. 2B) and TCM (Fig. 2C) were found in significantly higher numbers in the dLN and ndLN compared with WT animals. Therefore, reducing the functional avidity by lowering the availability of Lck enabled protection against the contraction of the response and its restriction to the lymphoid site most proximal to tumor Ag persistence. Moreover, low Lck-containing, LckON mice maintained a population of TCM cells that both persisted and recirculated and may have continuously fed conversion into the effector pool.

The expansion and persistence of TCM in tumor-bearing LckON mice was not particular to LACK-specific T cells as total CD44hi T cells were increased (Fig. 2D). It has been shown previously that transfer of comparable CD4 T cells from tumor-bearing animals was protective against tumor challenge, indicating that the tumor stimulates multiple specificities in addition to the 16.2b epitope (28). Subdivision of total CD44hi T cells into CD62Lhi (TCM) or CD62Llo (TE) showed that WT and LckON mice accumulated similar numbers of both when compared at day 12 after tumor development. At day 19, only LckON mice continuously accumulated memory-like cells despite the chronic Ag exposure. Thus, in animals expressing WT Lck, all tumor-responding CD4 T cells were consumed over time in the presence of persistent Ag, whereas they were continuously maintained in low Lck levels. A similar preference for maintenance of both TE and TCM populations in the presence of tumor was found for mice that lacked Lck altogether (LckOFF mice). Although fewer total tumor-responsive cells were generated during this time, they continued to accumulate rather than decline like WT cells, indicating that signals through Fyn were sufficient to drive differentiation of both these subsets of cells.

Low levels of Lck sustain proliferation in the face of chronic Ag
It was possible that the different numbers of memory-like cells found in WT and LckON mice reflected diverse proliferative
capacities of cells primed in the presence of high or low levels of Lck. It was shown previously (14) that the peak of the response to this tumor in WT BALB/c mice is at \( \sim 4 \) d after tumor challenge. Similar numbers of responding cells were recovered in WT and LckON mice at \( \sim 6–7 \) d after tumor challenge (Fig. 4A); to assess their proliferation, we measured the frequency of BrdU+ CD4+ T cells in the dLN and ndLN of tumor-bearing mice after continuous BrdU administration for the final 4 d before analysis. Seven days after tumor cell inoculation, comparable frequencies of BrdU+ CD4+ T cells were found in WT and LckON mice at \( \sim 6–7 \) d after tumor challenge (Fig. 4A); to assess their proliferation, we measured the frequency of BrdU+ CD4+ T cells in the dLN and ndLN of tumor-bearing mice after continuous BrdU administration for the final 4 d before analysis. Seven days after tumor cell inoculation, comparable frequencies of BrdU+ CD4+ T cells were found in WT and LckON mice (Fig. 4B), indicating that the rate of cycle between the two populations was similar at the start of the response. By day 14, many fewer WT cells had incorporated BrdU (4% compared with 13% at day 7 in the dLN), whereas the proliferation of LckON cells at day 14 was unchanged from day 7 (Fig. 4C). This sustained proliferation of LckON cells was dependent on Lck expression, because proliferation decreased in a cohort of mice that were taken off a Dox diet at day 7 and analyzed at day 14. Therefore, Lck being expressed at low levels supported long-term turnover of Ag-responsive cells and mitigated against the decline in self-renewing capacity, which occurred in cells expressing WT Lck. In naive mice, rates of BrdU incorporation were similar in WT and LckON mice (Fig. 4D), indicating that the background turnover rate of homeostatic CD4 memory cells is similar in these animals in the absence of overt antigenic stimulation.

It has been shown that IL-2 confers a survival advantage on CD4 T cells (29) and is important for T cell proliferation; therefore, we asked whether there was a difference in the production of IL-2 after immunization in vivo between LckON and WT animals. We immunized mice with LACK peptide pulsed dendritic cells (DCs) so that we could recall Ag-specific IL-2 producing cells at the peak of the response at day 5, as shown previously (27). Indeed, at this time there were substantially more peptide-specific CD4 cells with the potential to produce IL-2 in LckON mice (Fig. 5). Similar results were found at day 19 in tumor-bearing animals with more IL-2–expressing cells found in LckON mice (data not shown). Moreover, in LckON tumor-bearing mice we found increased numbers of cells ex-
pressing both CD25 and CD127 (data not shown), suggesting that more IL-2–responsive cells were able to enter the memory pool in these animals.

**Lck levels shape the phenotype of memory cells in a T cell-intrinsic manner**

In LckON mice, it was possible that T cells expressing low levels of Lck accumulated after priming, because of differences in environmental factors rather than as a consequence of intrinsic difference in the way in which TCR signals were integrated. If, for example, WT cells that were stimulated to become terminally differentiated effector cells were able to kill Ag-bearing APCs or engaged in fratricide or production of cytokines that affected the T cell differentiation or survival, it might negatively affect the generation of TCM. Alternatively, the growing tumor might dynamically change in the presence of different Ag-specific responses, in terms of clonality or secretion of suppressive cytokines, so that the emerging Ag-specific CD4 T cell population might be differentially affected in animals expressing different levels of Lck. To investigate whether cell-intrinsic versus environmental factors played a role, we generated mixed BM chimeras from donor 16.2b WT and 16.2b LckON mice. In this instance, homozygous 16.2b WT (H-2d) donors were used to distinguish them from LckON H-2b<sup>bd</sup> cells; because these were BM chimeras, the T cells arising from the two genotypes were tolerant of one another. The BMs were mixed in a 9:1 LckON:WT ratio, to generate approximately equal representation of the two cell types in the periphery, and injected into irradiated BALB/c<sup>Rag<sup>2</sup>−/−</sup> hosts (Fig. 6A). After 12 wk in the presence of Dox, reconstitution was confirmed by analysis of peripheral blood, and recipients with the periphery, and injected into irradiated BALB/c<sup>Rag<sup>2</sup>−/−</sup> hosts (Fig. 6A). After 12 wk in the presence of Dox, reconstitution was confirmed by analysis of peripheral blood, and recipients with

Staining with anti–H-2Kb Ab permitted identification of the phenotype of LACK-specific CD4 T cells expressing normal or low Lck levels at day 19 of tumor development in the same animal (Fig. 6B). Compared with naive controls, TS/A-LACK tumor-bearing chimeras accumulated LACK-specific CD4 T cells with TCM and TE phenotypes at high frequency (Fig. 6B, compare top left [naive] with lower left [tumor-bearing] panel). As before, LACK-specific CD4 T cells derived from cells expressing normal levels of Lck mainly differentiate toward a TE rather than a TCM phenotype (∼6:1 ratio, gated WT) (Fig. 6B, lower middle panel). In contrast, LACK-specific CD4 T cells derived from cells expressing lower levels of Lck maintained both TE and TCM phenotypes in approximately equal proportions (Fig. 6B, lower right panel), indicating that CD4 T cells expressing different levels of Lck behaved in the chimera as they did in separate animals.

Once again, injection of BrdU 2 d prior to analysis (Fig. 6C) showed that T cells from WT progenitors proliferated less by day 19 than T cells derived from LckON cells, confirming that cells expressing low levels of Lck do not exhaust their self-renewal

**FIGURE 4.** Sustained proliferation of LckON CD44<sup>hi</sup> CD4 T cells in tumor-bearing mice. A, Numbers of Tet<sup>+</sup> CD44<sup>hi</sup> cells recovered at days 7, 14, and 21 after tumor challenge of WT and LckON mice. B, Mice were given BrdU from day 3 of tumor challenge until day 7, and similar proliferation rates were seen among CD44<sup>hi</sup> cells in WT and LckON animals. C, By day 14 of tumor challenge, an equivalent 4-d BrdU pulse labeled many more dividing cells in LckON compared with WT mice. Dox withdrawal at day 7 reduced proliferation, indicating that proliferation relies on Lck expression. D, LckON and WT naive mice labeled for 4 d show equivalent BrdU incorporation. Data are representative of two independent experiments.

**FIGURE 5.** DC vaccination stimulates more IL-2–producing cells in LckON mice than in WT mice. Mice were challenged with LACK peptide-pulsed autologous BM-derived DCs 5 d before analysis. LN cells were restimulated for 6 h with LACK peptide-pulsed splenic APCs in the presence of brefeldin A before staining with tetramer and CD44 (top panel) or intracellular staining for IL-2 and IFN-γ (bottom panel). LckON mice showed a higher proportion of IL-2–staining cells than WT mice, indicating preferential expansion of this population in conditions of low Lck expression.
potential despite chronic Ag persistence. As before, long-term proliferative responses were dependent on continual expression of low Lck, because switching off Lck expression 14 d after tumor sacrifice at day 19. One group of mice had Dox removed from their diet at d7, the remainder stayed on Dox diet. All mice were given BrdU 2d before injection (ON dox → OFF dox) reduced turnover of LckON cells.

Cells with reduced Lck show less evidence of exhaustion
It was likely that the disappearance of the WT TCM population was due to exhaustion in the face of chronic Ag exposure. We reasoned that, even at early time points when recirculating CD4 T cells were found at comparable levels in the lymphoid organs of both WT and LckON tumor-bearing animals, WT cells with a TCM phenotype might already differ in the expression of exhaustion markers as a prelude to their early disappearance in response to systemic chronic Ag stimulation. Expression of a number of markers that correlate with exhaustion have been described, mainly but not exclusively in CD8 effector T cells, including CD127, KLRG1, PD-1, and Blimp-1. Thus, high expression of CD127 together with low expression of KLRG1 was inversely correlated with exhaustion in CD8 T cells (30), whereas PD-1 was shown to be upregulated early upon manifestation of exhaustion (31, 32). In addition, Blimp-1 was shown to be upregulated in exhausted CD8 T cells in vitro and in vivo (33, 34) and to be upregulated late in CD4 T cell differentiation (35, 36).

TCM phenotype cells from WT and LckON mice 11 d after tumor inoculation were sorted, and mRNA was analyzed by qPCR for expression of exhaustion markers. We consistently found that LckON TCM cells expressed significantly less KLRG1, PD-1, and Blimp1 than did WT cells (Fig. 7A), suggesting that although phenotypically TCM, WT cells are more terminally differentiated and prone to exhaustion than their LckON counterparts. Finally, we checked the expression levels of Caspase-3, a terminal effector protease required in apoptosis, and found that TCM cells from WT animals express higher levels of Caspase-3 mRNA than do LckON TCM-like cells, suggesting that cells that are chronically activated in the presence of higher levels of Lck are associated with activation-induced cell death.

CD127 expression has been linked to the survival of CD4+ memory cells (37), and in CD8 effector cells CD127 expression has been reported to indicate that these cells are destined to enter the TCM pool (38). However, no difference was found in the amount of CD127 on cells from WT or LckON mice with tumors either at early (11 d) or later (20 d) time points (data not shown). In addition, we monitored Bcl-2 expression and there was no difference in the proportion of CD44+ cells that expressed Bcl-2 nor in the levels of expression, either by intracellular staining or by qPCR (data not shown).

LckON CD4 T cells help control tumor growth
Tumor growth can be controlled by CD8 CTLs that mediate direct cytolytic activity against tumor cells. In the TS/A-LACK adenocarcinoma model, the tumor cells are not direct targets for CD4+-mediated killing, because they lack MHC class II expression and their parent line Tsi/A relies on CD8+ T cells to be rejected (39). However, treatment of tumor-bearing mice with anti-CD4 Ab was shown to result in increased tumor bulk (14), whereas adoptive transfer of tumor-specific CD4 cells conferred partial protection (28), indicating that CD4 helper cells were involved in restricting tumor growth. The 16.2ß rearranged Vß4 TCR transgene came originally from a CD4+ cell and may skew the repertoire of CD8 cells that develop in these animals. Therefore, to
address whether the CD4 memory cells that develop in tumor-bearing LckON animals had beneficial antitumor effects, we infused naive polyclonal CD8 T cells into tumor-bearing mice and looked for control of tumor growth.

16.2B LckON mice that received infusions of naive CD8 T cells had smaller tumors compared with WT 16.2B mice and 16.2B LckON mice that did not receive naive CD8 T cell infusion (Fig. 7B). Although the control of tumor growth tended to be transient, comparison of LN cells 1 wk after the last adoptive transfer with purified CD8 T cells. Data are representative of two independent experiments.

Discussion
The role of T cell avidity in the generation of effector and memory cells was addressed in this study by manipulating an intracellular kinase, Lck. We have shown previously that, although primary T cells can respond to TCR ligation in the absence of Lck if they express the related Src-family member Fyn, the threshold of triggering is raised substantially (20). In this study, we showed that the absence of Lck in LckOFF mice did not preclude formation of phenotypically distinct TCM and TE subpopulations in response to Ag exposure, but their numbers were limited by their impaired ability to expand. WT levels of Lck resulted in robust early expansion of TCM and TE, but in time these numbers reduced in the face of persistent Ag. In this context, neither too much nor too little signal seemed to be achieved with ~10% of normal Lck levels in LckON mice, which resulted in continuous accumulation of both TCM and TE with specificity for tumor-associated Ag.

In the interest of clarity, we defined the CD44hiCD62Llo population of Ag-responding cells as TE, given that tumor Ags were continually present, and they preferentially accumulated in the Ag-draining LN over time; however, this population could contain both effector cells and effector memory cells. Similarly, in mouse but not human studies, long-term central memory cells are generally defined as persisting in the absence of Ag, but because the tumors were not rejected we did not follow this population for a long term. However, LACK-specific cells with a CD44hiCD62Llo phenotype were generated after peptide-DC vaccination in both 16.2B and WT mice and were protective in the latter (27), suggesting this phenotype demarcates bona fide memory cells. Moreover, it has been suggested that long-lived central memory cells can be programmed from as early as the first cell division after Ag encounter (4), and our data are compatible with their early generation. Although our data do not directly address the relationship between TCM and TE that were generated to tumor Ags, it is clear that both subpopulations were detectable at similar initial frequency regardless of the strength of signal (i.e., whether Lck was high, low, or absent). Therefore, our data are not compatible with models that suggest that the signal strength per se directly influences whether a cell becomes a TE or TCM. In WT mice, decline of TCM by day 19 of tumor growth was mirrored by a drop in TE, which is consistent with either an early asymmetric fate decision (4) or a “fate commitment with progressive differentiation” type model of effector cell generation from a concomitantly generated, cycling TCM pool (3). In addition, the continual presence of both TCM and TE phenotype cells in LckON animals suggested that the former might be continuously seeding the latter.

We have shown previously that Lck influences multiple signaling pathways downstream of TCR engagement, but that not all pathways are equally dependent on Lck (20). For example, activation of PLCγ and Ca2+ mobilization were much more dependent on Lck than activation of the ERK/MAPK pathway. One consequence of these perturbations in signaling was that the production of IL-2 was reduced in LckON and LckOFF cells (20). More recently, we have found that the kinetics of IL-2 production is also influenced by reducing Lck in CD8 T cells (J. Kleczkowski, unpublished data) so that although less IL-2 is produced, production continues for much longer, up to 48 h in vitro. Generally, IL-2 production from naive T cells is transient (40), because the cytokine stimulates a negative feedback loop upon binding the IL-2R that shuts off IL-2 transcription (41). Therefore, lower levels of cytokine that might not activate the feedback loop could sustain IL-2 production, and prolonged IL-2 availability has been shown to promote sustained expansion, certainly for CD8 T cells (42, 43). In addition, IL-2 was shown to enhance CD4 T cell survival (29) and memory generation (44). Consistent with this finding, we found an increased proportion of LACK-specific cells expressing intracellular IL-2 in LckON animals after DC vaccination and in tumor-bearing mice. Moreover, there were...
many more cells in the LckON tumor-bearing mice, even at late time points that expressed both CD25 and CD127 consistent with an increased capacity for long-term survival. It has been suggested that one reason for the failure of the adaptive immune response to reject tumors is that T cells become exhausted in the face of persistent Ag. Our data are consistent with this explanation, as the loss of TCM in WT mice in response to tumor was correlated with an increased expression of markers associated with exhaustion that were readily detectable by 11 d after tumor inoculation. Moreover, the decline in the proliferative potential of WT cells after 2 wk in the presence of tumor further indicated their exhausted state. In contrast, cells expressing less Lck showed lower expression of molecules associated with exhaustion and terminal differentiation. It is noteworthy that BLIMP-1 expression has been shown to be associated with terminal differentiation (36) and to be inversely correlated with the ability to produce IL-2 (35), and the levels of BLIMP1 detected in LckON cells were close to naive cell levels. In addition, T cells with less Lck continued to cycle at similar rates throughout the period of tumor exposure in an Lck-dependent manner, indicating that they retained the ability to respond to Ag.

Our data raise the intriguing possibility that a potential therapeutic approach to improving or rejuvenating the immune response to persistent Ags, such as tumors, would be to try to lower the functional avidity (e.g., with a low dose Src-kinase inhibitor) to prevent exhaustion of the response. A similar suggestion was raised from the recent observations that rapamycin, an immunosuppressant that inhibits the mammalian target of rapamycin (mTOR) pathway, and metformin, an activator of AMP kinase, were able to promote better CD8 memory cell development (45, 46). We have shown that Lck levels affect PI3K signaling and more specifically phosphorylation of mTOR targets (47); therefore, manipulating Lck can target the same pathways as rapamycin. Our data also have implications for adoptive T cell therapies in which the focus is determined by signal strength. Nat. Immunol. 4: 355–360.


Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31: 296–308.


