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Cutting Edge: Tumor-Specific CTL Are Constitutively Cross-Armed in Draining Lymph Nodes and Transiently Disseminate to Mediate Tumor Regression following Systemic CD40 Activation¹

Philip A. Stumbles,^{2*} Robyn Himbeck,^{*†} Jeffrey A. Frelinger,[‡] Edward J. Collins,[‡] Richard A. Lake,^{*†} and Bruce W. S. Robinson^{2,*†}

The cross-arming of effector CTL in response to cross-presented tumor Ags is predicted to fail in the absence of CD40 stimulation. However, questions remain regarding the role of CD40 signaling and additional CD4⁺ T cell-derived signals in this process. To address this, we have analyzed the cross-arming of tumor-specific CTL effectors in vivo in a mouse model of established tumor and tumor regression following CD40 activation. We found that tumor-specific CTL were constitutively cross-armed in tumor-draining lymph nodes during tumor growth and that systemic CD40 activation did not alter CTL cross-arming in the tumor-draining lymph nodes. Rather, CD40 activation induced peripheral dissemination of tumor-specific CTL effectors that required continual CD40 stimulation to maintain peripheral CTL and tumor regression. These data indicate that CD40 activation enhances the peripheral survival of constitutively cross-armed CTL and that persistent CD4⁺ T cell signals are required for their long-term activity. The Journal of Immunology, 2004, 173: 5923–5928.

The cross-arming of naive CD8⁺ T cells to become effector CTL following recognition of cross-presented tumor Ag in lymph nodes is thought to be a key event that fails during the development of antitumor T cell immunity. Although the factors governing the efficiency of this process remain incompletely understood, CD4⁺ T cell-derived signals have been shown to play an important enhancing role, either at the point of CD8⁺ T cell activation in lymph nodes or in the periphery by preventing CTL deletion and aiding T cell traffic into tumors (1, 2).

The principal helper signal provided by CD4⁺ T cells for CTL activation was initially thought to be IL-2, aiding expansion and survival of activated CTL (3). More recently, it has

been shown that activation of cross-presenting dendritic cells (DC)⁴ through CD40 ligation can substitute for CD4⁺ T cell help in enhancing the efficiency of cross-presentation and CTL cross-arming in vitro (4–6). Activation of DC through CD40 is an NF- κ B-dependent event that results in up-regulation of costimulatory molecules and production of proinflammatory cytokines (7). CD40-activated DC have been shown to be potent activators of tumor-infiltrating CD8⁺ T cells in vitro (8) and to induce tumor-specific CTL that provide protective tumor immunity or eliminate established tumor (9). Anti-CD40 therapy has also been successful in vivo in the treatment of a variety of mouse tumor models in vivo (10–14). However, questions regarding the mechanism of CD40 activation on antitumor CD8⁺ T cell responses in vivo remain. First, is CD40 activation essential for the induction of effector CTLs in response to cross-presented tumor Ags? Second, what is the role of CD4⁺ T cell-derived molecules besides CD40 as cofactors in this response? Third, once specific CTL responses have been efficiently induced does the effect become independent of CD40?

We have addressed these issues in a mouse model of established solid tumor, the rejection of which has previously been shown to be CD4⁺ T cell dependent (2). We show that tumor-specific CTL effectors were constitutively cross-armed in vivo in tumor-draining lymph nodes (DLN) during tumor growth and that this was not enhanced by systemic CD40 activation. Rather, CD40 activation disseminated CTL peripherally to induce tumor regression independently of other CD4⁺ T cell-derived factors, an event that was a transient in the absence of continued triggering of CD40.

Materials and Methods

Mice

BALB/c and BALB/c congenic *nu*^{-/-} mice were purchased from the Animal Resources Centre (Canning Vale, Western Australia) and maintained under

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³ Abbreviations used in this paper: DC, dendritic cell; DLN, tumor-draining lymph node; HA, hemagglutinin; CLN, contralateral lymph node.

clean conditions. BALB/c mice expressing a transgenic TCR specific for the immunodominant H-2K^d-restricted peptide IYSTVASSL of A/PR8/8/34 (H1N1) influenza virus hemagglutinin (HA), termed CL4 TCR (10), were kindly provided by Dr. L. Sherman (The Scripps Research Institute, La Jolla, CA) and backcrossed to BALB/c for at least 12 generations. All experiments used female mice at 8 wk of age.

Tumor cell line maintenance and inoculation

The generation of the BALB/c-derived mouse mesothelioma cell line AB1 and transfection with the gene encoding influenza HA (AB1-HA) has been previously described (11). The AB1-HA line is MHC class I⁺, class II⁻, and CD40⁻. On day 0, 1×10^6 tumor cells in a volume of 100 μ l were injected s.c. into the right flank of recipients. Mice were euthanized when tumors reached 10 mm², which was usually by day 21 of tumor growth.

mAbs and tetramers

The rat IgG2a anti-mouse CD40 agonistic mAb FGK.45 (kindly provided by Dr. A. Rolink, University of Basel, Basel, Switzerland) was purified from tissue culture supernatant by protein G-Sepharose adsorption (Amersham Biosciences, Piscataway, NJ) and 100 μ g of purified IgG in 200 μ l of PBS injected i.v. every second day as indicated in the text from day 14 of tumor growth. Initial experiments showed that normal rat IgG given under a similar dosage regime had no significant effect on tumor growth rates at any time point or on in vivo CTL activity in peripheral lymph nodes ($p = 0.39$) or spleen ($p = 0.54$) at day 21 of tumor growth when compared with PBS; therefore, 200 μ l of PBS i.p. was chosen as a control for all experiments. The YTS.169 (rat anti-mouse CD8 α) and YTS.191 (rat anti-mouse CD4) mAbs were purified from tissue culture supernatant and 100 μ g of purified IgG injected i.p. every second day, starting at day -2 of the experiment and continued to coincide with anti-CD40 mAb treatment. PE-conjugated anti-mouse CD44 and CD62L were from BD Pharmingen (Sydney, Australia). CL4/H-2^d PE-tetramers were generated as

previously described (12) and used in conjunction with cell surface staining with anti-mouse CD8 α -FITC. Surface fluorescence was assessed by flow cytometry (FACScan; BD Biosciences, Mountain View, CA) by analyzing a minimum of 5×10^3 CD8 α ⁺ gated events.

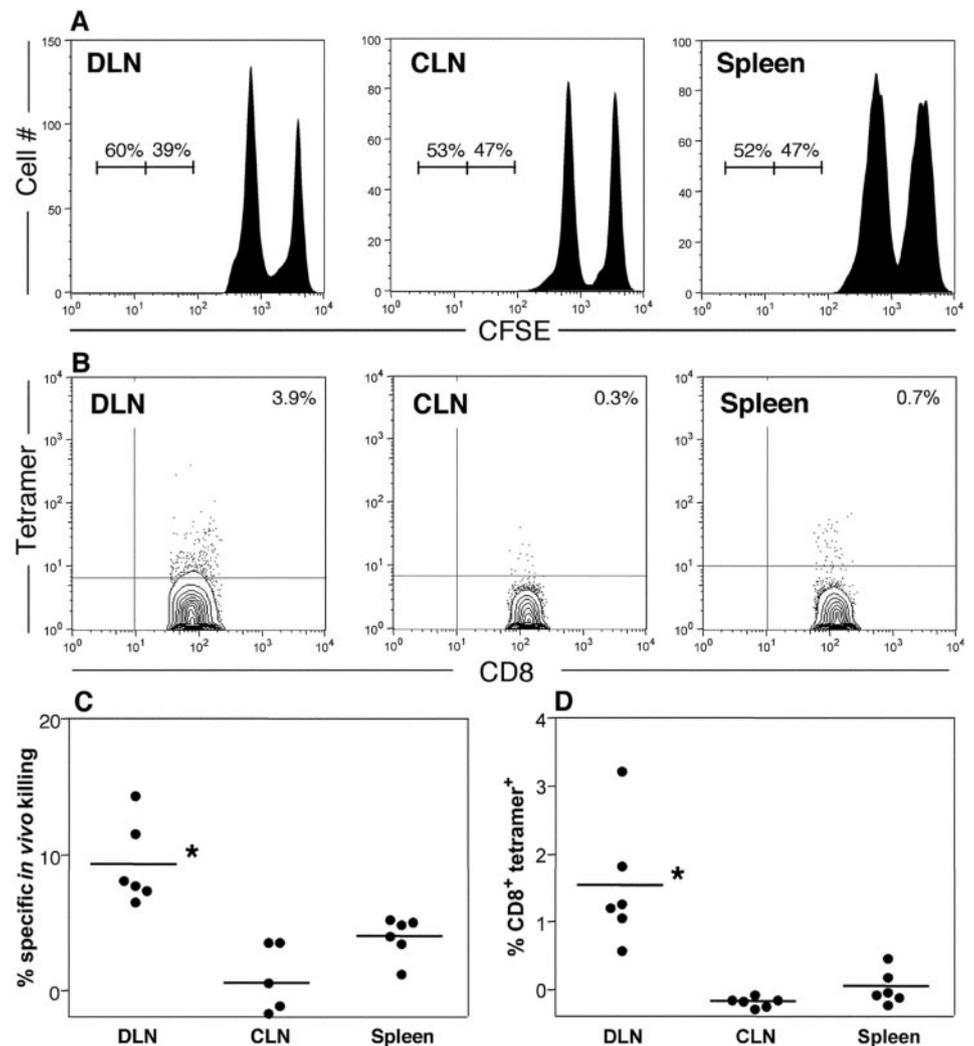
In vivo CTL assay

Analysis of tumor Ag-specific effector CTL activity in vivo was performed by an adaptation of the method of Oehen et al. (13) as previously described (11). Briefly, BALB/c spleen cells were resuspended in PBS and divided into two equal populations, one of which was labeled with the CL4 peptide at a concentration of 1 μ g/ml for 90 min at 37°C. The cells were then labeled with CFSE (Molecular Probes, Eugene, OR) at a final concentration of 5 μ M for peptide-pulsed cells (CFSE^{high}) and 0.5 μ M for nonpulsed cells (CFSE^{low}). Viable cells were isolated and mixed at a ratio of 1:1 and a total of 2×10^7 cells were injected i.v. into recipient animals. DLN, contralateral lymph nodes (CLN), and spleens were then harvested 24 h after adoptive transfer and CFSE fluorescence intensity was analyzed by flow cytometry (FACScan; BD Biosciences). Results are expressed as percentage lysis of the Ag-pulsed peak as compared with the internal nonpulsed reference peak.

In vivo tumor Ag presentation assay

MHC class I- or class II-restricted tumor-associated HA presentation was assayed in vivo by using the "Lyons-Parish" assay of the in vivo proliferation of adoptively transferred tumor-specific T cells as previously described (11, 14). Briefly, lymph node cells from TCR transgenic mice were labeled with CFSE and 1×10^7 cells injected i.v. into control or anti-CD40 mAb-treated recipient mice at day 21 of tumor growth. Three days following adoptive transfer, DLN or CLN were harvested and CFSE intensity was analyzed by flow cytometry. To assess the extent of CD8⁺ T cell activation in anti-CD40 mAb-treated or control mice, DLN from recipients of CFSE-labeled CL4 T cells were harvested as

FIGURE 1. Tumor-specific CTL are constitutively cross-armed in the DLN during tumor growth. *A* and *C*, BALB/c mice were inoculated with AB1-HA tumor cells on day 0 and then assayed for in vivo killing of CL4-peptide loaded spleen cell targets in the DLN, CLN, and spleen on day 14 of tumor growth as described in *Materials and Methods*. *, $p = 0.002$ vs CLN and 0.009 vs spleen. *B* and *D*, DLN, CLN, and spleen from BALB/c mice inoculated with AB1-HA were harvested on day 14 of tumor growth and stained with FITC-labeled anti-CD8 α and PE-labeled CL4/H-2K^d tetramer. *, $p = 0.002$ vs CLN and spleen. Quadrant marker positions were based on an isotype control mAb for CD8 and unlabeled CD8⁺ T cells for tetramer.



above and additionally labeled with anti-mouse CD44-PE or anti-mouse CD62L-PE before FACS analysis.

Statistical analysis

All statistical determinations were by two-tailed nonparametric (Mann-Whitney) *t* test with 95% confidence intervals. Only significant values are indicated.

Results and Discussion

Tumor-specific CD8⁺ T cells are constitutively cross-armed in the DLN during tumor growth

It has previously been suggested that ineffective CTL responses to tumor-associated Ags are a result of lack of recognition of cross-presented Ag (ignorance) or cross-tolerance of CD8⁺ T cells (15, 16). To examine this, we used a mouse model of established solid tumor expressing a transfected *neo* Ag (AB1-HA) and examined effector CTL activity using an *in vivo* assay that is a direct measure of the endogenous effector CTL population rather than of the precursor CTL population usually measured by *in vitro* expansion assays. The AB1-HA tumor is CD40⁻ and nonimmunogenic (as defined by failure of an irradiated lysate to protect against rechallenge), retaining similar *in vivo* growth characteristics as the parental AB1 tumor line (11). Surprisingly, significantly elevated levels of HA-specific effector CTL activity were detected *in vivo* in the DLN compared with the CLN ($p = 0.009$) and spleen ($p = 0.002$) of mice bearing established tumors on day 14 following inoculation in the absence of any evidence of systemic effector CTL activity (Fig. 1, *A* and *C*). Consistent with this, CL4/H-2K^d tetramer staining was also significantly elevated in the DLN but not CLN ($p = 0.002$) or spleen ($p = 0.002$) of mice bearing established

AB1-HA tumors (Fig. 1, *B* and *D*). Importantly, these data indicate that the cross-priming events were not manifest systemically but rather remained restricted to the DLN, i.e., had systemic measures of CTL effector function been relied upon, these important local events would have been missed. Our previous studies confirmed that tumor Ag is cross-presented by a bone marrow-derived APC (12) and not directly presented by tumor cells in the DLN: the current data confirm that this is a productive event, i.e., ignorance of Ag or CTL cross-tolerance were not found. In addition, these data confirm that the CL4 epitope is available for cross-presentation and that its delivery into the cross-presenting pathway is not restricted, as has recently been described for epitopes located within the signal sequences of exogenous proteins (17).

CD40 activation does not boost activation of tumor-specific CD8⁺ T cells in the DLN of tumor-bearing mice

Although in the current model CTL effector activity was detected in the DLN of tumor-bearing mice, this was of a relatively low level when compared with the *in vivo* HA-specific CTL activity following a strong stimulus such as influenza virus (70–80% HA-specific *in vivo* CTL activity 4 days postinoculation *i.p.*; data not shown). As CD40 ligation has previously been shown to induce CD8⁺ T cell activation and tumor killing (18), we next examined whether this could similarly increase CD8⁺ T cell activation in the DLN of AB1-HA tumor-bearing mice. Purified naive HA-specific CD8⁺ TCR transgenic T cells were labeled with CFSE and transferred into

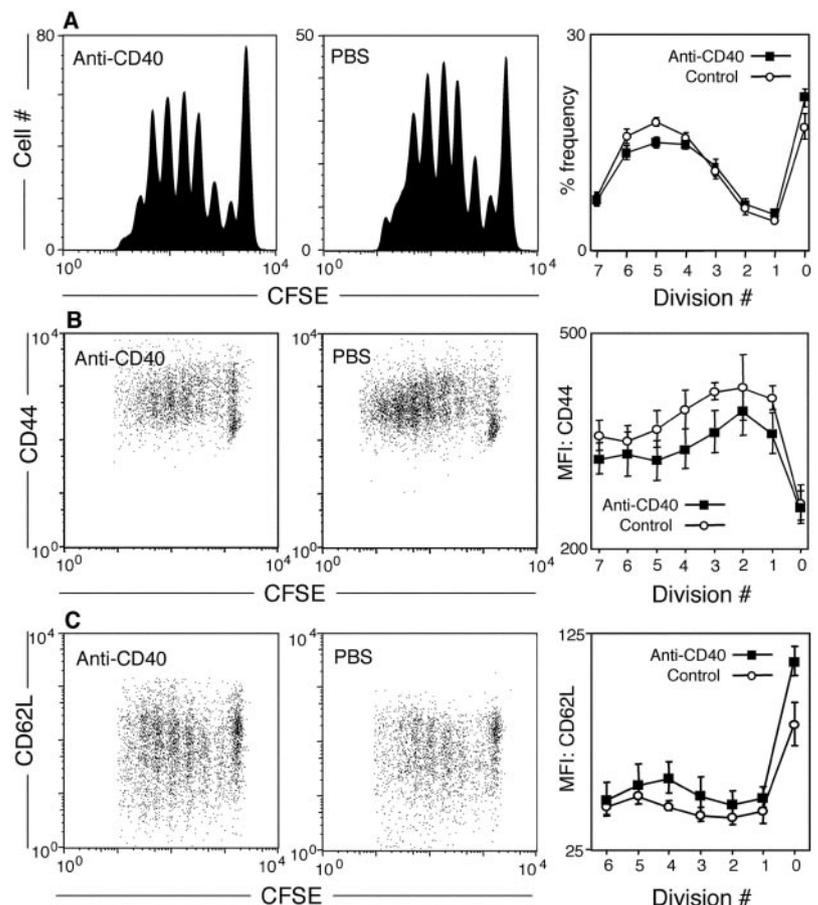


FIGURE 2. Cross-arming of tumor-specific CD8⁺ T cells in the DLN of tumor-bearing mice is not enhanced following CD40 activation. BALB/c mice were inoculated with AB1-HA tumor cells on day 0, then treated with anti-CD40 mAb or PBS on days 14, 16, and 19. On day 21, 1×10^7 CFSE-labeled, naive CD8⁺ CL4-TCR transgenic T cells were adoptively transferred into tumor-bearing recipients and DLN cells were harvested 3 days later and stained for CD8 in conjunction with CD44 or CD62L. CFSE⁺CD8⁺ cells were then analyzed by flow cytometry for (*A*) cell division, (*B*) CD44, and (*C*) CD62L expression.

AB1-HA tumor-bearing mice following anti-CD40 mAb treatment and examined for cell proliferation and expression of cell surface activation markers. Surprisingly, despite inducing tumor regression (see Fig. 4A), CD40 activation had no significant effect on naive CD8⁺ T cell activation in the DLN, as indicated by the equivalent levels of tumor-specific CD8⁺ T cell division (Fig. 2A) and increases in CD44 (Fig. 2B) and decline of CD62L expression (Fig. 2C) on dividing cells in anti-CD40 mAb treated and control tumor-bearing mice. Consistent with a lack of enhanced CD8⁺ T cell activation, CD40 ligation did not increase in vivo CTL activity (Fig. 3C; DLN) or numbers of CL4/H-2K^d tetramer-staining CD8⁺ T cells (Fig. 3D; DLN) in DLN despite the fact that Ag was continually cross-presented to host CD8⁺ T cells and the dose of CD40 mAb was sufficient to induce tumor regression. These data contrast with the “altered” activation phenotype seen in CD8⁺ T cells responding to cross-presented HA from normal tissues, which were not cross-armed and eventually deleted (19), and suggest that APC entering the DLN in this system are fully functional in their capacity to cross-arm CTL. This is also in contrast to other systems where CD40-mediated activation of DC is required for cross-priming of tumor-specific CTL (8), although it should be emphasized that most of these studies have used in vitro expansion assays,

i.e., they are measuring in vitro expansion of CTL precursors, not in vivo CTL effector function.

CD40 ligation disseminates CTL to peripheral sites by a mechanism independent of disseminated Ag

In contrast to the findings in the DLN, effector CTL activity was significantly elevated in peripheral lymphoid organs following CD40 ligation, as shown by elevated in vivo CTL activity in the CLN ($p = 0.002$) and spleen ($p = 0.002$) (Fig. 3, A and C). Similarly, numbers of CL4/H-2K^d tetramer-staining CD8⁺ T cells were also significantly elevated in the CLN ($p = 0.002$) of anti-CD40 mAb-treated mice (Fig. 3, B and D) similar to the effect described by van Mierlo et al. (20). CD40-CD154 interactions have been shown to be a potent signal in promoting the migration of DC from peripheral sites to the lymph node (21), raising the possibility that CD40 activation may be disseminating Ag-bearing DC. To test this, CFSE-labeled HA-specific CD8⁺ TCR transgenic T cells were transferred into tumor-bearing, anti-CD40 mAb-treated animals at day 21 of tumor growth and examined for proliferation in CLN 3 days later. An equivalent lack of cross-presentation of Ag to naive CD8⁺ T cells in peripheral LN of anti-CD40 mAb-treated and control

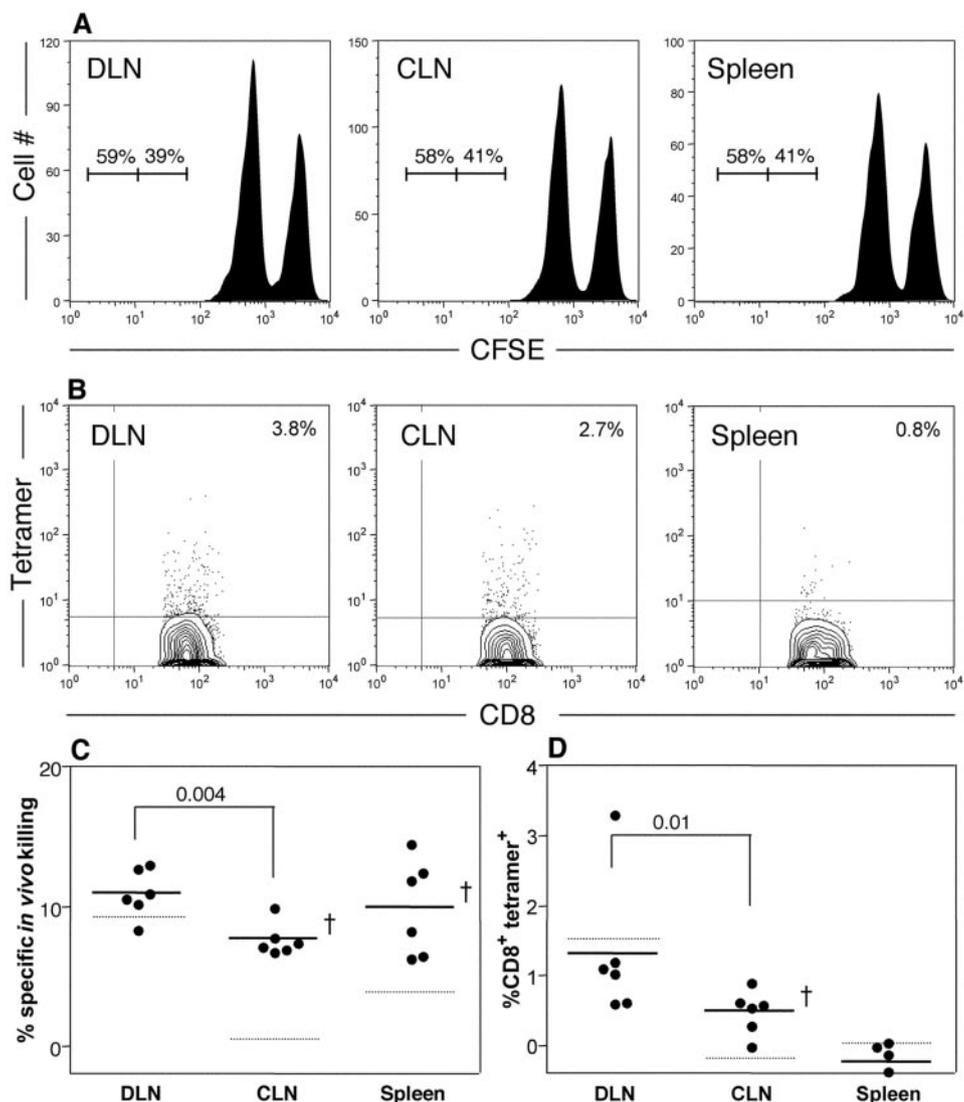


FIGURE 3. CD40 activation peripherally disseminates tumor-specific CTL but does not enhance CTL cross-arming in the DLN. BALB/c mice were inoculated with AB1-HA tumor cells on day 0 and treated with anti-CD40 mAb on days 14, 16, and 19. On day 21, in vivo CTL activity (A and C) and CL4/H-2K^d tetramer staining assessed in DLN, CLN, and spleen as described for Fig. 1. Dotted lines indicate mean values for in vivo CTL and tetramer data obtained from control PBS-treated tumor-bearing mice. †, $p = 0.002$ for CLN and spleen of anti-CD40 mAb-treated compared with control PBS-treated tumor-bearing mice.

mice was observed, demonstrating that neither Ag nor Ag-bearing APC were induced to disseminate following anti-CD40 mAb treatment (data not shown). Thus, the increased CTL activity and CD8⁺ T cell numbers we observed in the periphery were not a result of CD40-induced priming of CD8⁺ T cells in multiple sites. Although we cannot formally exclude direct activation of CD40 on CD8⁺ T cells (22), this is unlikely given that CD40 activation had no significant effect on CD8⁺ T cell priming in DLN. Rather, these data suggest that CTL were constitutively cross-armed in the lymph node draining the tumor and that CD40 activation of cross-presenting APCs drives more cross-primed CD8⁺ T cells into the periphery. We have preliminary data to suggest this may operate through increased resistance to CD152-mediated deletion in the periphery (data not shown).

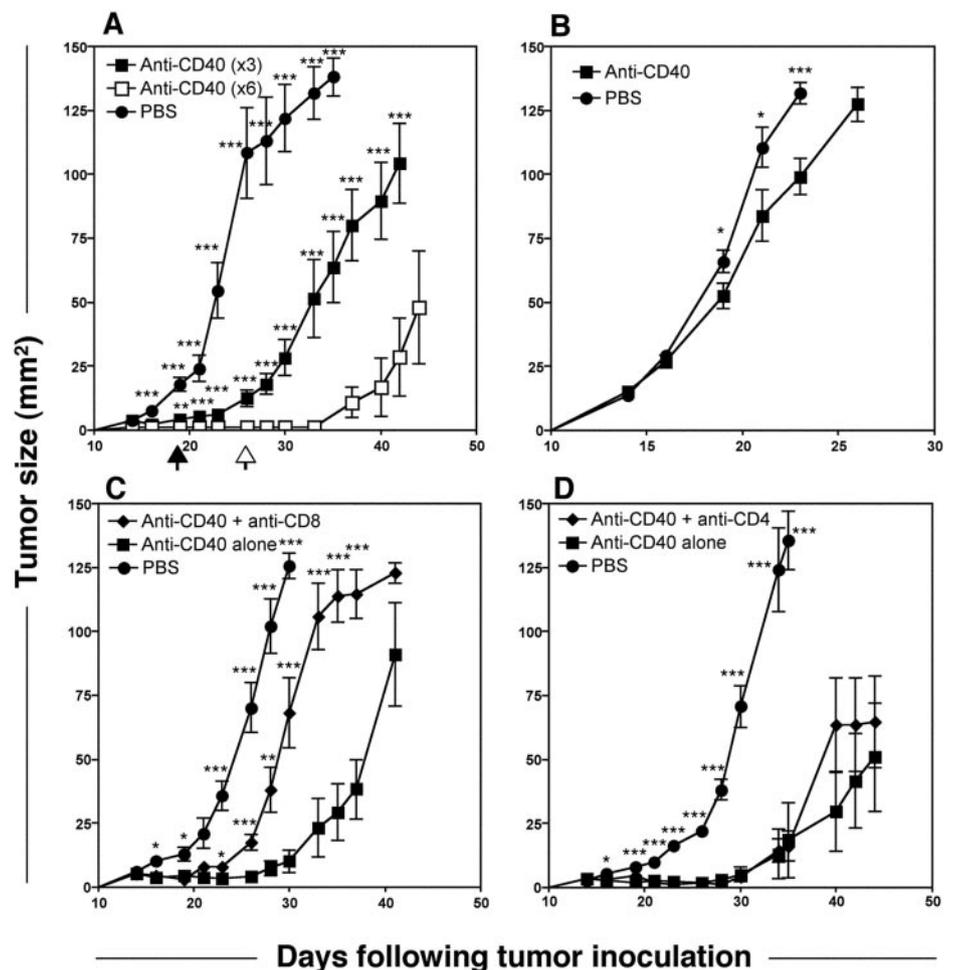
CD40 activation-induced CD8⁺ T cell-mediated tumor regression is dependent on sustained CD40 signaling but independent of other CD4-derived signals

Although the provision of CD4⁺ T cell “help” for CD8⁺ T cells is a goal of tumor immunotherapy, one concern is that these CD4⁺ T cells may persist in the host and induce anti-self responses. Therefore, we analyzed the duration of CD40-mediated effects on tumors following the initial CD40-induced tumor regression. Unexpectedly, control of tumor growth was effective only during the period of anti-CD40 mAb treatment and 7–9 days following cessation of therapy (Fig. 4A, day 19, solid arrow), after which tumors regrew at normal rates. To fur-

ther determine whether this was related to CD40 administration, we prolonged anti-CD40 mAb treatment for a further 7 days. Again, tumor growth was controlled for the period of therapy after which tumors regrew at normal rates (Fig. 4A, day 26, open arrow). Consistent with an association between CTL dissemination and tumor regression, a decline in disseminated *in vivo* CTL activity and tetramer staining was observed 10 days following cessation of anti-CD40 mAb therapy, a time point at which tumors in all mice had begun to regrow (data not shown). The inability to induce sustained tumor regression was consistent with studies showing short-term alloreactive CD8⁺ T cell activation following CD40 activation required CD4⁺ T cell-derived IL-2 to be sustained (23) and suggests that although CD40 activation alone can substitute for tumor-specific CD4⁺ T cells in the short-term, long-term sustained antitumor CTL responses may be dependent on the activation of tumor Ag-specific memory CD4⁺ T cells.

Anti-CD40 mAb was not effective in controlling tumor growth in these mice, although a small effect was seen at late time points, confirming that an intact T cell compartment was required and that the anti-CD40 mAb effects were not due to direct effects on tumor cells or vasculature (Fig. 4B). CD8⁺ T cell depletion (Fig. 4C) also reduced the effectiveness of anti-CD40 mAb therapy, indicating that tumor regression was CD8⁺ T cell dependent. The abrogation was not complete, however, suggesting a minor role for other cells in this process.

FIGURE 4. CD40 activation induces tumor regression dependent on sustained CD40 signaling. *A*, BALB/c mice were inoculated with AB1-HA tumor cells on day 0, then treated with 100 μ g of anti-CD40 mAb or PBS on three (days 14, 16, and 19) or six (days 14, 16, 19, 21, 24, and 26) occasions and tumor growth was monitored. Arrows indicate the day of last mAb treatment for three (closed arrow) or six (open arrow) treatments. Values of *p* are shown for PBS vs anti-CD40 $\times 3$ and anti-CD40 $\times 3$ vs $\times 6$ (*B*) BALB/*c*^{nu/nu} mice were inoculated with AB1-HA tumor cells on day 0 then treated with anti-CD40 mAb or PBS on days 14, 16, and 19 and tumor growth monitored. *C*, BALB/c mice were inoculated with AB1-HA tumor cells on day 0 then treated with either CD8-depleting mAb on days 12, 14, 16, and 19 in combination with anti-CD40 mAb on days 14, 16 and 19 or with anti-CD40 mAb alone or PBS on days 14, 16, and 19. Values of *p* are shown for PBS vs anti-CD40 + anti-CD8 and anti-CD40 + anti-CD8 vs anti-CD40 alone (*D*) BALB/c mice were inoculated with AB1-HA tumor cells on day 0 then treated with either anti-CD4 depleting mAb on days 12, 14, 16, and 19 in combination with anti-CD40 mAb on days 14, 16, and 19 or with anti-CD40 mAb alone or PBS on days 14, 16, and 19. Values of *p* are shown for PBS vs anti-CD40 + anti-CD4 and PBS vs anti-CD40 alone. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001.



In contrast, CD4⁺ T cell depletion had no effect (Fig. 4D), indicating that other CD4⁺ T cell-derived signals were not essential cofactors in this process.

In conclusion, the use of *in vivo* methods of analysis of CTL effector function demonstrate that tumor-draining APC do not require additional CD40 activation to cross-arm CTLs and that dissemination of cross-armed CTLs is a rate-limiting step for tumor regression. Such observations would have been missed if systemic analyses of CTL function, which have been used in almost all other published studies, had been used. These data also highlight the need for future tumor immunotherapy approaches based on CD40 activation to continuously deliver the CD40 agonist during the treatment period to maintain peripheral antitumor CTL effector function.

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