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T Cell Immunity to Lymphoma Following Treatment with Anti-CD40 Monoclonal Antibody

Alison L. Tutt, Lyn O’Brien, Akmal Hussain, Graham R. Crowther, Ruth R. French, and Martin J. Glennie

In this study we demonstrate that treatment with anti-CD40 mAb eradicates a range of mouse lymphomas (BCL1, A31, A20, and EL4), but only when used against i.v. tumor doses in excess of $10^7$ cells. Only partial protection was seen against smaller tumor loads. We saw no evidence that anti-CD40 mAb changed the phenotype of the lymphomas or inhibited their growth in the initial period following treatment, but it did result in a rapid expansion of cytotoxic CD8+ cells that was able to clear the neoplastic disease and provide long-term protection against tumor rechallenge. The CTL responses were blocked by mAb against a range of coreceptors and cytokines, including CD8, B7-1, B7-2, LFA-1, and IFN-γ, but not CD4 or CTLA-4, indicating the presence of a conventional cellular Th1 response. Furthermore, we found evidence of cross-recognition between lymphomas (BCL1 and A20) as measured by cytotoxicity and IFN-γ responses in vitro and using tumor rechallenge experiments, suggesting common target Ags. Finally, although anti-CD40 was shown to stimulate NK cell killing, we could find no role for these cells in controlling tumor growth. These data underline the ability of anti-CD40 mAb to potentiate CTL responses and the potency of cellular immunity in eradicating large quantities of syngeneic tumor. The Journal of Immunology, 2002, 168: 2720–2728.

 Despite the recent clinical success of certain “naked” mAb, such as anti-CD20 and anti-Her2/neu, most workers continue to look to cellular immunity to provide effective treatment against malignant diseases (1–3). The continued conviction that cellular immunology holds the most potential for cancer treatment has been reinforced over the last decade by the identification of numerous human tumor Ags that appear sufficiently “foreign” for recognition by T cells, and the isolation of tumor-infiltrating lymphocytes from biopsies that are capable of recognizing and killing cells expressing these new targets (2, 3). Furthermore, the presence of tumor-infiltrating lymphocytes is generally considered a good prognostic factor for clinical outcome, and they have also shown encouraging therapeutic application when expanded ex vivo in IL-2 and then administered to autologous cancer patients (4). To date considerable evidence points to CD8+ CTL as the responding effector cells in patients, with by far the majority of the cancer epitopes identified to date being expressed on MHC class I. While the relative numbers of MHC I- and II-restricted tumor epitopes is still under investigation, the disproportionate number of identified CTL epitopes has led to the suggestion that T cell responses may be ineffectual in part due to inadequate CD4 T cell help and that this might be remedied with treatments that boost or replace CD4 T cells (5).

CD40 is a member of the TNFR superfamily that plays a critical role in both humoral and cellular immune responses (6). It was initially shown to be constitutively expressed on the majority of B cells but has since been found on a wide range of cells, including epithelial and endothelial cells and on all APC. The natural ligand for CD40, CD154, is a trimeric TNF-like molecule that is expressed mainly on activated Th cells (6) and appears to provide an important pathway by which T cells can control immune responses via the APC. For example, several groups have shown that ligation of CD40 on dendritic cells (DC)3 is a potent method of improving the Ag-presenting function of these cells and of empowering them to present Ag to naive CD8 cells (7–9). Such conditioning or licensing of DC can be achieved with soluble CD40 ligand (CD40L) and CD40 mAb, which are both able to provide sufficient crosslinking activity to stimulate the CD40-expressing cells. While we still have much to learn about the cellular responses to CD40 ligation, the more obvious changes include increased expression of adhesion and costimulatory molecules, such as ICAM-1, CD80/CD86, and CD40, and increased production of inflammatory cytokines such as IL-12, IL-18, and macrophage-inflammatory protein-1α (10). Results such as these support the view that CD40 stimulation promotes a pattern of cytokine production that skews toward a Th1-type immune response (11). Importantly, CD40 mAb and soluble CD40L are already showing evidence of clinical potential, being able to potentiate immune responses in a number of therapeutic settings, including T cell responses to peptides and proteins (12, 13), infectious disease (14, 15), and treating neoplastic disease in animals and patients (16–19).

We have recently shown that anti-CD40 mAb can stimulate extremely potent CTL responses against syngeneic murine lymphomas (16). These responses eradicate the existing tumors, leave mice resistant to rechallenge, and bypass the need for T cell help. In this study we extend this work to define the nature of the CD8 responses and investigate their specificity in a range of mouse lymphoma models.

Tenovus Research Laboratory, Cancer Sciences Division, University of Medicine, Southampton General Hospital, Southampton, United Kingdom

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2 Address correspondence and reprint requests to Dr. Martin J. Glennie, Tenovus Research Laboratory, Cancer Sciences Division, University School of Medicine, Southampton General Hospital, Southampton SO16 6YD, U.K. E-mail address: mjg@soton.ac.uk.

3 Abbreviations used in this paper: DC, dendritic cell; CD40L, CD40 ligand; anti-asialo GM1; anti-ASGM1.
Materials and Methods

Animals and cell lines

BALB/c, CBA/H and C57Bl/6k mice were all supplied by Harlan (Blackthorn, Oxon, U.K.) and maintained in local animal facilities. BCL1 (20) and A31 (21) mouse B lymphoma lines were maintained by in vivo, i.p. passage in BALB/c and CBA mice, respectively. Spleens were removed at the terminal stage of disease and single cell suspensions were prepared as described previously (22). rBCLs, cells are a subtype derived from BCL1, which grow in culture (23). A20, EL4, and YAC cell lines (all American Type Culture Collection, Manassas, VA) were maintained in culture using standard medium (see below).

American Type Culture Collection, Manassas, VA) were maintained in 10% of total cells, which anti-CD40 mAb or an isotype-matched control (i.v.) on the day when the culture using standard medium (see below).

Antibodies

mAbs used in this study were M17/5.2 (anti-LFA-1), 16-10A1 (anti-B7-1), GL-1 (anti-B7-2), R4-6A2 (anti-IFN-γ), M5/114.15.2 (anti-MHC class II), 34-1-2S (anti-MHC class I, K, and Dα), all obtained from the American Type Culture Collection. In addition, 3/23 (anti-CD40) was a gift from G. Klaus (National Institute of Medical Research, London, U.K.) (24); ID3 (anti-CD19) was provided by D. Fearon (University of Cambridge School of Clinical Medicine, Cambridge, U.K.) (25); and K9-18 (anti-K6) and 19-191 (anti-iκβ) were kindly provided by D. Kiousis (National Institute of Medical Research) and YTA3.1.2 (anti-CD4) and YTS 169 (anti-CD8) were from S. Cobbold (Sir William Dunn School of Pathology, Oxford, U.K.) (26). The anti-B cell receptor mAb Mc10-6A5 (anti-BCL1, IgG) and Mc39-12 (anti-IgG1) were prepared in house and have been described previously (27).

Anti-idiotype GM1 (anti-ASGM1) antiserum for NK cell depletion was from Wako (Neuss, Germany).

Hybridoma cells were expanded in stationary culture using 5% supplement-free DMEM and their IgG mAb prepared by precipitation with saturated ammonium sulfate followed by fractionation on protein A or G (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. Four of the rat mAbs (Mc10-6A5, M5/114.15.2, YTS169, and YTA3.1.2) were prepared by ion-exchange chromatography on DEAE (Whatman, Clifton, NJ) as described previously (28). The purity of all IgG preparations was checked by electrophoresis (Beckman EP system; Beckman Coulter, Palo Alto, CA) and HPLC using a Zorbax GF250 Bio Series column (Jones Chromatography, Hengoed, U.K.) (29).

Immunotherapy

Groups of age-matched mice were injected i.v. with tumor cells (BCL1, A31, A20, or EL4) on day 0 and, unless stated otherwise, treated with mAb daily from day 2 or 3 onwards indicated for each experiment. In vivo T cell depletion was as described by Cobbold et al. (26), using i.p. injection of 0.5 mg of anti-IgD mAb (YTS 169) and/or 1 mg of anti-CD4 mAb (YTA3.1.2). The injections were repeated every 4–5 days, or as indicated, and the effectiveness of depletion was confirmed by flow cytometry on PBL.

Flow cytometric analysis of splenic lymphocytes following tumor and anti-CD40 mAb

To follow changes in splenic lymphocytes, groups of age-matched BALB/c mice were given 5 × 10³ BCL1 cells on day 0 (i.v.) and then 1 mg of anti-CD40 mAb or an isotype-matched control (i.v.) on the day when the level of tumor cells in the spleen had reached 5–10% of total cells, which was typically day 4–6 post-tumor. To ensure that the tumor load was in the range at this time of treatment, parallel groups of mice were assessed for Id⁺ splenocytes by flow cytometry on a FACSCalibur (BD Biosciences, Mountain View, CA) using PE-anti-CD19 and FITC-anti-CD1, IgD. Changes in CD4⁺ and CD8⁺ lymphocytes were followed using FITC-anti-CD4 and PE-anti-CD8. Knowing the yield of spleen cells (normal plus tumor) and the percentage of each subset from the flow cytometric analysis we were able to estimate the total yield of each type of cell. In experiments to look at the effect of various blocking mAbs on the response to anti-CD40 mAb treatment, 0.5 mg of the blocking mAb was injected i.p. on the same day as the injection of anti-CD40, and then again on days 3 and 4 after the anti-CD40 mAb.

Cytotoxicity assays

A standard 4-h ⁵¹Cr release assay (30) was used to assess cytotoxic activity of splenic effectors (NK or cytotoxic CD8). Splenic homogenates were prepared from mice and, when necessary, any contaminating tumor cells were removed using FITC-anti-Id mAb, followed by anti-FITC MACS beads and LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) as described in the manufacturer’s instructions. The remaining effector cells were washed and resuspended at 2 × 10⁶ cells/ml before being diluted for mixing at the required ratio with ⁵¹Cr-labeled target cells (final volume of 200 μl) in a U-bottom culture plate (Life Technologies). In experiments to determine the blocking activity of Abs, mAbs were included at a final concentration of 50 μg/ml. The plates were centrifuged at 200 × g for 5 min at room temperature, incubated for 4 h at 37°C in a CO₂ incubator, and then centrifuged at 500 × g for 5 min before finally harvesting 100 μl of the supernatant to estimate ⁵¹Cr release. All determinations were performed in triplicate. The maximum release of radioactivity was calculated using target cells to which 150 μl of 1% Nonidet P-40 had been added. The per-cent of specific ⁵¹Cr release was calculated using the standard formula: percentage of specific release = ([sample release – background release]/(maximum release – background release)) × 100.

Rechallenge of immune splenocytes with tumor in vitro

Splenocytes were prepared from mice that had previously been cured of BCL1, lymphoma by anti-CD40 mAb between days 30 and 50 post-initial tumor challenge. A total of 5 × 10⁵ cells were then mixed with 5 × 10³ rBCL1 or other BALB/c tumor cells as stimulators in a final volume of 200 μl (supplemented RPMI 1640) in a U-bottom culture plate and plates were centrifuged at 200 × g for 5 min at room temperature before incubating for 48–72 h at 37°C in a CO₂ incubator. The plates were then centrifuged at 500 × g for 5 min before harvesting the supernatant to determine the level of IFN-γ release by ELISA as described below.

ELISA for IFN-γ and IgM idiotyp"
unculled mice in this treated group survived for >70 days and were resistant to rechallenge with fresh BCL1. An alternative ELISA format was also used to show that the loss of serum IgM Id was not the result of class switching to IgG (data not shown).

We next asked whether a large tumor dose, given as the initial inoculum and treated early, could also be controlled with anti-CD40 mAb. Fig. 2 shows the results obtained when groups of mice were given between $10^5$ and $10^7$ A31 i.v. on day 0, followed by four mAb treatments on days 3–6. Surprisingly, we consistently found that as the tumor dose was increased so the efficacy of treatment improved. Thus at the lowest cell dose ($10^5$ A31), the anti-CD40 mAb provided ~25 days protection, while at the high cell-dose ($10^7$), all mice survived beyond 100 days (defined as long-term survivors). To investigate the generality of this phenomenon, we repeated similar experiments with A31 and three other lymphomas, BCL1, A20, and EL4. EL4 is a CD40– T cell lymphoma, while the other tumors are all CD40+ B cell lymphomas.

The results in Fig. 3 show that in each tumor the larger the tumor inoculum the more effective the anti-CD40 mAb treatment. This observation was particularly striking in experiments performed in the EL4 tumor, because it expresses the CD4 T cell marker and thus interfered with such an analysis. We have shown previously that, at least in the case of BCL1, anti-CD40 mAb treatment of tumor-bearing mice promotes the rapid expansion of CD8 lymphocytes at the time the mAb is given. The explanation may be provided if the CTL response is directed at a scarce or weak tumor Ag that only reaches a threshold detection level for recognition when the tumor is growing in the lymphoid system at high doses.

Anti-CD40 mAb promotes a CD8+ T cell response in lymphoma-bearing mice

In three B cell models we next followed changes in tumor and T cells (CD4 and CD8) following anti-CD40 mAb treatment by flow cytometry. This investigation was not possible in the case of the EL4 tumor, because it expresses the CD4 T cell marker and thus interfered with such an analysis. We have shown previously that, at least in the case of BCL1, anti-CD40 mAb treatment of tumor-bearing mice promotes the rapid expansion of CD8 cells and does not result in any detectable humoral response in the serum of anti-CD40 mAb–“cured” mice as measured by flow cytometry. Interestingly, we found no marked change in the growth rate of these B cell tumors during the initial period following the anti-CD40 mAb treatment despite the expression of CD40. Fig. 4A shows that for at least 4 days after the mAb the growth rate of the tumor in the control and the anti-CD40 mAb-treated animals is essentially the same, and the anti-CD40 mAb did not promote any marked change in the surface phenotype of CD40+ lymphoma cells in vivo, with the exception that CD40 became blocked and modulated by the treatment (data not shown). It therefore appears that the therapeutic activity of the mAb cannot be explained by a direct cytotoxic effect on the tumors. The capacity of this treatment to deal with bulk

**FIGURE 1.** Response of BCL1 lymphoma to late treatment with anti-CD40 mAb. Groups of five mice were given $10^5$ fresh BCL1 cells i.v. as indicated and then monitored for tumor development by ELISA for serum IgM Id as described in Materials and Methods. On days 16–19 mice received 0.5 mg of either irrelevant IgG (Control, □) or anti-CD40 mAb (anti-CD40, ■). The symbols and error bars indicate the mean and SD for the five mice in each group.

**FIGURE 2.** Anti-CD40 mAb treatment of A31 lymphoma shows improved efficacy with higher tumor inoculates. Groups of five mice were given $10^5$, $5 \times 10^5$, $10^6$, or $10^7$ A31 cells on day 0 as indicated in the key. They were then treated on days 3–6 with 500 μg/mouse/day of control IgG (filled symbols) or anti-CD40 (open symbols) mAb and monitored for tumor development.

**FIGURE 3.** High inoculates of CD40+ and CD40– lymphoma are cured by anti-CD40 mAb treatment. Groups of five mice were treated as shown in Fig. 2 with the four different lymphomas and the number of cells shown. The data were plotted to show the median survival for each group of control IgG (open bars) and anti-CD40 mAb (filled bars)-treated mice. All experiments where concluded at 100 days, when mice were defined as long-term survivors. This experiment shows that as the tumor inoculum is increased survival of the control mice becomes shorter, as expected, but, surprisingly, survival of anti-CD40 mAb-treated mice is extended. These data are representative of between two and four similar experiments for each lymphoma.
Data represent one of three similar experiments.

were monitored by duplicate spleens were harvested and tumor cells, CD4 cells, and CD8 cells (mAb, on the days indicated (arrows). From day 3 or 4, or control IgG (Œ), lymphoma cells on day 0 and then treated with 1 mg of mAb, anti-CD40 (Œ), /H11003 treatment. Groups of mice were inoculated i.v. with 5 \times 10^7 fresh lymphoma cells before the therapeutic activity takes over and the cell number starts to decline (Fig. 4A). At this point FACS analysis shows that up to half of the splenic lymphocytes will be tumor. The results in Fig. 4B show that in all three B cell lymphoma models treatment was followed by a sharp decline in CD8 cells was preceded by an equally rapid loss of CD8 cells were harvested and tumor cells, CD4 cells, and CD8 cells were monitored by flow cytometry as described in Materials and Methods. Data represent one of three similar experiments.

tumor is underlined by the fact that in these three models the tumor load can reach up to 5 \times 10^8 cells/spleen before the therapeutic activity takes over and the cell number starts to decline (Fig. 4A). At this point FACS analysis shows that up to half of the splenic lymphocytes will be tumor. The results in Fig. 4B show that in all three B cell lymphoma models treatment was followed by a sharp but short-lived rise in CD8 cells (Fig. 4B), starting \sim 2 days after mAb treatment, which peaked at 4–6 days after treatment and then subsided over a period of between 24 and 48 h. In each case the decline in CD8 cells was preceded by an equally rapid loss of splenic tumor cells (Fig. 4A). Similar, but less exaggerated, changes were seen in CD4 T cells (Fig. 4C). This is consistent with T cell depletion studies which show that removal of CD8, but not CD4, cells in these models with anti-CD8 or anti-CD4 mAb ablates the therapeutic activity of the anti-CD40 mAb (Ref. 16 and data not shown). Interestingly, these studies also showed that when BCL1 or A31 cells were inoculated without anti-CD40 mAb (Δ) we consistently observed a small but significant rise in CD8 cells, and although these responses were obviously unable to control tumor development, they do indicate immune recognition of these syngeneic tumors. Thus the current data support the idea that the anti-CD40 mAb treatment boosts a weak, but preexisting, CD8 tumor response that is achieved without the need for CD4 Th cells.

Next we performed blocking studies to investigate which coreceptors and cytokines were important in these T cell responses and to define some of the molecular recognition involved. For this work we concentrated on the BCL1 model mainly because it generated the most profound changes in CD8 cells following anti-CD40 treatment. During this investigation blocking mAb were given against LFA-1, B7-1, B7-2, and IFN-γ. In each case the blocking mAb prevented the response and the BCL1 tumor grew progressively. The results in Fig. 5A show the number of splenic tumor cells quantified each day following treatment and blocking with anti-B7-1 and anti-B7-2. Interestingly, blocking with either or both anti-B7-1 and anti-B7-2 mAb completely prevented an effective response against the growing tumor (Fig. 5A). This lack of response was reflected in the failure of CD8⁺ T cells to expand in the normal manner (Fig. 5B). In fact, when both anti-B7.1 and anti-B7.2 mAb were administered, CD8⁺ cell numbers fell below the normal level. Fig. 5, C and D, also shows that blocking mAb to LFA-1 and IFN-γ were effective in inhibiting the therapeutic activity of anti-CD40 mAb. Together these results point to the fact that CD8 T cells respond against BCL1 using conventional adhesion and coreceptor molecules and appear to generate a Th1 response. These same mAb were also found to inhibit the activity of anti-CD40 mAb in immunotherapy survival studies using BCL1 and A31 (data not shown). Interestingly, mAb against ICAM-1 and CTLA-4 failed to block the CD40 mAb-induced responses either...

FIGURE 4. Changes in tumor (A), CD8 (B), and CD4 (C) cells in B lymphoma-bearing (BCL1, A31, or A20) mice following anti-CD40 mAb treatment. Groups of mice were inoculated i.v. with 5 \times 10^7 fresh lymphoma cells on day 0 and then treated with 1 mg of mAb, anti-CD40 (Δ), or control IgG (Œ) mAb, on the days indicated (arrows). From day 3 or 4, duplicate spleens were harvested and tumor cells, CD4 cells, and CD8 cells were monitored by flow cytometry as described in Materials and Methods. Data represent one of three similar experiments.

FIGURE 5. Blocking of the anti-CD40-mediated antitumor response with mAb to B7-1, B7-2, IFN-γ and LFA-1. Groups of mice were given 5 \times 10^7 BCL1, cells on day 0 and treated with anti-CD40 mAb (long arrow) on day 6. Blocking or control mAb (0.5 mg/dose/mouse) was given i.p. on days 6–9 (arrowheads). The key indicates the experimental groups. For each group, splenic tumor cells, CD8⁺ T cells, and CD4⁺ T cells were monitored by flow cytometry as described in Materials and Methods. The total numbers of splenic tumor (A and C) and CD8 (B and D) cells are shown for blocking of B7-1 and B7-2 (A and B) and IFN-γ and LFA-1 (C and D). Each data point represents the mean of duplicate spleens; data represent one of three similar experiments.
Anti-CD40 mAb promotes an NK cell response

Previous work has shown that anti-CD40 mAb can promote therapeutic NK cell responses against murine tumors (32, 33). Three experimental conditions were investigated to clarify the contribution of tumor cells and anti-CD40 to the NK cell response: in the first group, mice were given just the BCL1 cells (5 x 10^5) on day 0; in the second, mice received anti-CD40 alone on day 4; and in the third, mice received 5 x 10^7 BCL1 cells i.v. on day 0 and then anti-CD40 on day 4 when the splenic tumor load was ~5%. The results in Fig. 6 show that in all three settings mice responded with a wave of increased NK cell cytotoxic activity against 51Cr-labeled YAC targets which lasted ~3 days and peaked between 24 and 48 h after mAb treatment. Interestingly, just giving the tumor cells alone was sufficient to promote some increase in NK cell activity against YAC cells (Fig. 6A). We do not have an explanation for this observation, but it was a consistent finding and again points to immune recognition of neoplastic cells. However, it is important to note that these mice did not develop an appreciable cytotoxic response against BCL1 cells. In contrast, mice given either anti-CD40 mAb alone (Fig. 6B) or BCL1, and anti-CD40 mAb (Fig. 6C) did make a small but consistent response against BCL1 that peaked at ~48 h after anti-CD40 mAb treatment. This early cytotoxic response to the BCL1 was not mediated by CTL because it could not be blocked by anti-CD8 mAb (Fig. 6C, open bars). Finally, the major difference in mice treated with tumor and anti-CD40 mAb (Fig. 6C) was that they made a striking cytotoxic response against the BCL1 tumor at 96 h after the anti-CD40 mAb treatment. Furthermore, this second burst of killing was important because, unlike the early cytotoxic activity, it was blocked by anti-CD8 mAb and thus was mediated by CD8+ CTL. These results show that following tumor inoculation, with or without anti-CD40 mAb, mice respond with a rapid burst of NK cell activity capable of killing YAC cells but unable to control these syngeneic lymphomas. However, in those mice given both tumor and anti-CD40 mAb, the NK cells are followed at ~4 days by CTL activity capable of controlling the disease. This latter T cell killing also coincides with the increase in CD8 T cells seen in Fig. 4. Finally, we performed in vivo NK depletion using anti-ASGM1 antiserum and confirmed that NK cells were not required for the therapeutic activity of anti-CD40 mAb, at least in the BCL1 model (data not shown). This depleting Ab did not reduce the rapid rise in CD8 T cells seen in the spleens of responding mice, although we did sometimes note both an acceleration of tumor growth in anti-ASGM1-treated mice and a slight delay of ~24 h in the initiation of the CTL response. Overall we can conclude that NK cells are not an essential element in the anti-CD40 mAb-induced response.

**CD8+ T cells provide long-term protection to anti-CD40 mAb-cured mice**

We next established that mice (BALB/c, CBA, or C57Bl/6) cured of syngeneic B or T cell lymphomas by anti-CD40 mAb treatment acquire long-term immunity against rechallenge with the same tumors. The data for all four models are summarized in Table I. Tumor-bearing mice were treated with anti-CD40 mAb and then left for an extended period, 40 to >150 days, before i.v. rechallenge with 10^5 cells from the same tumor that was used in the original therapy. All mice were appreciably protected compared with naive controls; however, the data clearly show that resistance to tumor, which was initially very high, declines with time. For example, 67% of mice that had been treated for the BCL1 lymphoma 40 days previously were fully protected (>100 days) when rechallenged, while only 12% of mice rechallenged between 80 and 149 days after initial treatment showed the same degree of resistance. Interestingly, the EL4 tumor, which was very dependent on tumor load when treated with anti-CD40 mAb and did not respond at all to small tumor inocula (10^5) (Fig. 3), proved the most robust in terms of long-term memory. Thus all mice (17 of 17) that survived for 60 and 79 days after first having the tumor were completely resistant to rechallenge. Furthermore, even after they had been left for >150 days, two-thirds were still able to resist tumor rechallenge.

Although previous studies have shown that anti-CD40 mAb treatment of mouse lymphoma initiates a CD4-independent, CD8-dependent antilymphoma response, we wished to investigate whether the effector phase of a memory response showed a similar dependency. In these experiments, mice that had survived >40 days following BCL1 inoculation and anti-CD40 mAb treatment were first depleted of CD4 and/or CD8 T cells with mAb and then rechallenged in the normal way. The results in Fig. 7 show very clearly that, in contrast to CD8 depletion, depletion of CD4 cells before rechallenge did not significantly diminish the level of protection. Hence these data make a strong case for a CD8 T cell memory that provides long-term protection in the absence of a CD4 response.
**CD8⁺ T cell responses are cross-protective between lymphoma models**

Immune splenocytes taken from mice during their response to BCL₁ are cytotoxic for this tumor and for A20 (16). In these experiments we inoculated mice with $5 \times 10^7$ BCL₁ on day 0 and then treated them on day 4 with 1 mg of anti-CD40 mAb. On day 8 or 9, when the tumor was cleared but while the effector CD8⁺ T cells were still high, splenocytes were harvested and assayed for cytotoxic activity against a range of BALB/c tumor targets. In addition, a similar group of mice were left until days 35–50 to measure the IFN-γ response to the BALB/c tumors. The two assays could not be conducted simultaneously due to the high background levels of IFN-γ production during the early post-anti-CD40 mAb period. Fig. 8 shows the results of such an experiment. As expected, the BCL₁ tumor target itself initiated good killing and IFN-γ responses; however, similar responses were also stimulated by a second B cell tumor, A20. The level of IFN-γ production stimulated by the A20 in these experiments was somewhat variable and in the example shown did not result in a detectable increase (Fig. 8B). Overall these data indicated considerable cross-reactivity between A20 and BCL₁. Interestingly, other available BALB/c tumors were not recognized in these assays. Blocking studies with anti-MHC class I, -MHC class II, -CD4, and -CD8 mAb confirmed that the killing responses were mediated by CD8 T cells within the immune splenocytes (Fig. 8C). Finally, to confirm that this in vitro cross-recognition extended to cross-protection in vivo, we next performed rechallenge immunotherapy with BCL₁ and A20. Groups of mice were inoculated with either BCL₁ or A20 ($5 \times 10^7$ cells/mouse) and treated with anti-CD40 mAb in the normal way to cure the tumor. After 40–71 days they were rechallenged i.v. with $10^7$ cells from either the same or the alternative BALB/c tumor and then monitored for disease development. The results in Fig. 9 clearly show that, as with the in vitro assay, these two tumors provide almost complete cross-protection, confirming the recognition of common CD8⁺ T cell Ags.

**Discussion**

The belief that cellular rather than humoral immunity offers the best prospect of treating neoplastic disease has spawned numerous investigations into different, often vaccine- or DC-based, methodologies for promoting T cell responses. However, it is now also clear that, at least in model systems, Ab and soluble receptors offer a very convenient and potent means of promoting anticancer cellular responses; however, similar responses were also stimulated by a second B cell tumor, A20. The level of IFN-γ production stimulated by the A20 in these experiments was somewhat variable and in the example shown did not result in a detectable increase (Fig. 8B). Overall these data indicated considerable cross-reactivity between A20 and BCL₁. Interestingly, other available BALB/c tumors were not recognized in these assays. Blocking studies with anti-MHC class I, -MHC class II, -CD4, and -CD8 mAb confirmed that the killing responses were mediated by CD8 T cells within the immune splenocytes (Fig. 8C). Finally, to confirm that this in vitro cross-recognition extended to cross-protection in vivo, we next performed rechallenge immunotherapy with BCL₁ and A20. Groups of mice were inoculated with either BCL₁ or A20 ($5 \times 10^7$ cells/mouse) and treated with anti-CD40 mAb in the normal way to cure the tumor. After 40–71 days they were rechallenged i.v. with $10^7$ cells from either the same or the alternative BALB/c tumor and then monitored for disease development. The results in Fig. 9 clearly show that, as with the in vitro assay, these two tumors provide almost complete cross-protection, confirming the recognition of common CD8⁺ T cell Ags.

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![Figure 7](image-url)  
**FIGURE 7.** The role of CD4 and CD8 in mediating protection to tumor BCL₁ rechallenge. BALB/c mice were challenged with $5 \times 10^7$ BCL₁ cells/mouse and treated with anti-CD40 on day 4. They were then left for 40–60 days to ensure that antitumor immunity had been established (untreated mice develop lymphoma in 12–15 days). Groups (9–13 per group) of these “immune” animals were then treated with control IgG (A), anti-CD4 (B), anti-CD8 (C), or anti-CD4 plus anti-CD8 (D) mAb 24 h before rechallenge with $10^5$ fresh BCL₁, and subsequently every 4–5 days throughout the experiment to deplete the appropriate T cell populations. Tumor development was monitored daily and a group of naive mice injected with the same batch of BCL₁ tumor cells are included for comparison (E).

<table>
<thead>
<tr>
<th>Days Since Initial Challenge</th>
<th>BCL₁</th>
<th>A31</th>
<th>A20</th>
<th>EL4</th>
</tr>
</thead>
<tbody>
<tr>
<td>40–59</td>
<td>12/18 (67)³</td>
<td>11/15 (73)</td>
<td>14/20 (70)</td>
<td>ND</td>
</tr>
<tr>
<td>60–79</td>
<td>11/19 (58)</td>
<td>7/12 (58)</td>
<td>9/12 (75)</td>
<td>17/17 (100)</td>
</tr>
<tr>
<td>80–149</td>
<td>7/58 (12)</td>
<td>14/25 (56)</td>
<td>3/17 (18)</td>
<td>4/5 (80)</td>
</tr>
<tr>
<td>&gt;150</td>
<td>0/22 (0)</td>
<td>6/21 (29)</td>
<td>0/12 (0)</td>
<td>6/9 (67)</td>
</tr>
</tbody>
</table>

³ Mice that had been cured of the various lymphomas using anti-CD40 mAb were rechallenged with the same tumor. For the initial cure, mice were given $5 \times 10^7$ tumor cells with BCL₁, A31, and A20, but $2 \times 10^7$ with EL4, and then treated with anti-CD40 mAb as described in Materials and Methods. They were then left for the number of days indicated before being rechallenged.  
⁴ Shown in parentheses are percentages of cured mice showing full protection, which is defined as animals surviving beyond day 100 on i.v. rechallenge with $10^7$ fresh tumor cells.

**FIGURE 8.** Specificity of cytotoxic anti-BCL₁ CD8 T cells. Splenocytes were taken from mice on day 8 or 9 after they had received $5 \times 10^7$ BCL₁ cells/mouse and 5 days after anti-CD40 mAb (1 mg/mouse) at the peak of the T cell response and assessed for their cytotoxic activity. They were used without in vitro culture as effectors against a panel of six BALB/c tumors in standard 51Cr release assays at an E:T ratio of 150:1 (A), and against 51Cr-labeled BCL₁ cells in the presence of blocking mAb (50 µg/ml) with the specificities shown (C). Parallel experiments were performed to measure IFN-γ release using splenocytes taken from mice that had been treated 35–40 days earlier with BCL₁ and CD40 mAb. At this point the spontaneous level of IFN-γ had subsided, allowing any increases to be measured. Splenocytes were cultured with the same BALB/c tumors as above for 48 h (B). The level of IFN-γ in the supernatants of duplicate cultures was then assessed by ELISA (mean result plotted). Data represent one of three similar investigations.
Cross-protection between the BALB/c tumors, BCL1 and A20. Groups of BALB/c mice were challenged with A20 (■) or BCL1 (□) (5 × 10⁵/mouse) and treated with anti-CD40 mAb (1 mg/mouse) on days 3 and 4, respectively. On days 40–71, surviving mice were rechallenged i.v. as indicated with 10⁵ fresh BCL1 (A) or A20 (B) and monitored for tumor development as usual. For comparison, naive mice were also challenged with the same batch and dose of each tumor (▲). These data represent one of two similar experiments.

Immune splenocytes taken from responding mice were cytotoxic toward the tumor in vitro. This activity was provided mainly by CD8 T cells and was achieved without further stimulation in culture. The BCL1-reactive T cells also recognized A20 and provided cross-protection, which allowed mice cured of BCL1, or A20 to reject both these tumors when rechallenged. Interestingly, such cross-recognition did not extend to a range of other, nonlymphoid, BALB/c tumors, suggesting that the recognized Ag(s) was restricted to lymphocytes. Studies are under way to define these Ags. Finally, treatment of BCL1 with anti-CD40 mAb, and even the BCL1 lymphoma itself, promoted a wave of NK cell activity as measured by increased killing of YAC cells. However, we found no evidence that this played an important role in preventing lymphoma development or in curing the mice, because NK cell deletion using a polyclonal Ab (anti-ASGM1) did not block the efficacy of the CD40 mAb. Others have found CD40 stimulation with mAb or CD40L can up-regulate NK cell cytotoxic activity (32, 33), and that, at least in some models, these can account for a major component of the therapeutic activity. The most likely explanation for such activity is through CD40-promoted production of Th1 cytokines such as IL-12 and IFN-γ.

It is now well accepted that anti-CD40 mAb can act as a powerful adjuvant to promote vaccination. For example, CD40 ligation has been shown to promote CTL responses to peptide and protein Ags with known epitopes (12, 13), and functioned therapeutically against tumors where the targets were not defined (16, 18). It appears that in most situations these are Th1-skewed responses and that they occur without CD4 T cell help. Lefrancois et al. (13) used an OT-1 adoptive transfer system to show that agonistic anti-CD40 mAb was highly effective at promoting expansion and cytotoxic activity in CD8⁺ OT-1 cells when immunized with poorly immunogenic soluble OVA. The response was CD4 T cell-independent and required expression of CD40 on non-T cells, which, although not defined, were probably APC. Likewise, Diehl et al. (12) have shown that a tolerogenic response to a tumor-derived peptide could be converted to an immunogenic response simply by including anti-CD40 mAb. Again this work points to the APC, probably DC, as the target for the anti-CD40 mAb, in that its agonistic effects were still fully functional in B cell-deficient mice.

The effect of CD40 stimulation on APC, such as DC, has been extensively studied, showing that it acts by up-regulating costimulatory molecules and proinflammatory cytokines, thus equipping them to prime naive CTL (10). During normal cellular responses it appears that virus infection of APC or interaction of APC with CD40L (CD154)-expressing CD4 T cells are two mechanisms that equip APC to prime CTL (39). Thus CD4 T cells have the potential to initiate an immune response not only through the production of particular types of cytokine but also by way of activating signals delivered to APC. A number of groups (7–9) have models have similar requirements. The blocking activity of both anti-B7-1 and -B7-2 mAb was somewhat surprising, especially in view of the redundant role these two molecules appear to play in T cell responses. One possible explanation is that, despite performing the same costimulatory function, both are required to achieve an effective response against very weak tumor Ags. Thus blocking either B7-1 or B7-2 with mAb is enough to reduce the levels of costimulation below a minimum threshold required for the response. A second possible explanation is that mAb, including anti-B7-1 and anti-B7-2 (BCL1, and A31 lymphoma express these two Ags), that reacts with the lymphoma cells will perturb the T cell responses, for example, simply by altering the way in which tumor cells are processed by APC or the way the tumor cells present their own Ags.

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shown that anti-CD40 mAb or soluble trivalent CD154 can substitute for CD4 help to produce APC capable of presenting Ag effectively to naive T cells and thereby provide a similar stimulatory function to cognate CD4 helper cells. We propose that similar changes in APC are occurring when anti-CD40 mAb is used to treat splenic lymphoma in vivo. In all three B cell lymphoma models (BCL1, A31, and A20) presented in this work we found that the immune responses generated by CD40 mAb treatment were dependent on CD8 CTL and bypassed the need for CD4 T cell. At least in the BCL1 model, it appears that CD4 cells are also not required in the effector phase of the response because immune animals were still able to resist tumor rechallenge even following CD4 T cell depletion (Fig. 7). Because the tumor cells express surface CD4, the EL-4 T cell lymphoma could not be investigated with anti-CD4-depleting mAb in these studies. Thus it appears that CD40-reactive mAb is able to replace the need for a CD4 T cell response to prime CTL, as might occur in conventional antitumor CD8 T cell response. Such results point to a lack of endogenous CD4 tumor epitopes in these tumors and are consistent with the view that antitumor CTL responses are often thwarted due to the lack of suitable recognition by CD4 T cells. For example, Surman et al. (40) found that by adoptively transferring CD4+ T cells specific for a model tumor Ag they could promote the production of endogenous CD8+ CTL in the tumor-bearing mice and that these CTL were specific for the same model Ag. In addition, activation of APC provides the potential to cross-prime tumor Ags from the lymphomas into the MHC class I pathway of the APC. Although previously it was felt that Ags entering APC from outside the cell would only enter the MHC class II pathway, it is now clear that under certain conditions exogenous Ags can be delivered into the class I pathway for presentation into the CTL. The existence and importance of such a system with tumor Ags in vivo is still controversial, but it would provide a satisfactory explanation for the CTL responses seen with CD40− tumors, such as EL-4, in this work and against solid tumors described previously (18).

Growing evidence shows that the anatomical site in which tumors develop is very important for their immune recognition, with tumors that develop in the lymphatics being more inclined to induce immunity compared with those growing outside the immune system, such as under the skin (41). Recent work from Ochsenbein et al. (41) has shown for a range of tumors that the immune system is more likely to respond effectively in cases where the tumor cells “seed” into lymph nodes or grow in the spleen, while those tumors that remain confined to a s.c. site are often ignored by the immune system. Even immunogenic tumors, such as those transfected with viral genes, when grown s.c. promote quite weak responses. All four of the lymphomas used in this study are considered nonimmunogenic or only weakly immunogenic and would not be expected to stimulate ablative T cell responses. This is particularly the case for BCL1 and A31, which cannot be grown in vitro and have to be maintained by in vivo passage. However, in each case the tumors grow, at least initially after inoculation, as dispersed cells in the spleens surrounded by the immune system. Under such circumstances it appears that even the slightest immune response is sufficient for effective boosting by anti-CD40 mAb. The best evidence we have of immune recognition comes from the observation that T cell numbers, especially CD8+ cells, rise slightly after tumor injection (Fig. 4) and NK cell cytotoxic activity is promoted by a large i.v. inoculum of BCL1 (Fig. 6). Although it is not possible to grow these lymphomas at remote sites without their rapid migration to the spleen, we have recently found that anti-CD40 mAb treatment of large quantities of BCL1, in the peritoneal cavity is less effective than that seen against splenic tumors. This result is consistent with the idea that anti-CD40 mAb-induced immunity is more effective in situations where the immune system is in close proximity to the neoplastic cells. Likewise, we find that anti-CD40 mAb is relatively ineffective against solid tumors grown s.c. (18).

Recent results from this laboratory showed that anti-CD40 mAb had only a modest therapeutic effect on the colorectal carcinoma, CT26, grown s.c. and only in the case of a highly immunogenic carcinoma, CMT93, did it demonstrate appreciable effectiveness. Thus it appears that with highly immunogenic tumors anti-CD40 mAb will boost the immune response regardless of anatomical site, but with nonimmunogenic tumors such responses are seen only when the tumor is surrounded by the immune system.

Such a suggestion would help explain why we only see good tumor protection when treatment is initiated against a large tumor dose, this being achieved either with a large inoculum or by allowing sufficient time for in vivo tumor development before treatment. This would be consistent with a situation where the Ag load was limiting. The cross-reactive nature of the protection between BCL1 and A20, the two BALB/c lymphomas, is consistent with a limited antigenic expression. We have also found in both these tumors that the T cell repertoire in mice cured of tumors by anti-CD40 mAb is skewed in their TCR Vβ family usage (G. Crowther, C. Patrick, P. Johnson, R. French, and M. Glennie, manuscript in preparation), consistent with recognition of a limited number of common tumor (lymphoma) Ags.

The current findings have important implications for human immunotherapy, showing that strong CD8 CTL responses can be generated without excessive toxicity. A major advantage with such a strategy is that the responses are potentially directed at multiple tumor Ags and are therefore less likely than single target Ags to suffer escape variants, and they can be induced without identifying the target Ags. Recently, Vonderheide et al. (19) have shown interesting results in patients using a soluble CD154 fusion protein which in phase I trials showed acceptable toxicity and some encouraging clinical responses. The question of whether it will be more effective to use a human (or humanized) IgG anti-CD40 and run the risk of generating anti-Id responses or to use a soluble recombinant ligand, such as a CD154 trimer, remains to be seen. In this respect recently work has shown that a dodecamer of CD154 is far more active than trimeric CD154 at activating cells via CD40 (42).

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References


