Cyclophosphamide Inhibition of Anti-CD40 Monoclonal Antibody–Based Therapy of B Cell Lymphoma Is Dependent on CD11b+ Cells

Jamie Honeychurch, Martin J. Glennie, and Timothy M. Illidge

Abstract
Monoclonal antibody (mAb)–based immunotherapy is now established as an important option for treating some cancers. The antitumor effects may be further enhanced by combining mAb with conventional chemotherapy. Certain novel immunomodulatory mAbs such as anti-CD40 have shown significant activity in preclinical models. We therefore assessed the efficacy of combining anti-CD40 mAb, known to elicit CTL responses against murine lymphoma models with the commonly used cytotoxic drug, cyclophosphamide. Using the syngeneic tumor model, BCL1, we have shown that timing of cyclophosphamide relative to mAb is critical to therapeutic outcome. Pretreatment with cyclophosphamide 7 to 10 days prior to mAb results in markedly reduced survival outcome, similar to that achieved with cyclophosphamide alone. Conversely, when anti-CD40 is given before cyclophosphamide, the level of tumor protection was moderately increased. In vivo tracking experiments reveal that pretreatment with cyclophosphamide leads to diminished CTL expansion, as well as an increased number of CD11b+ cells that display an activated phenotype. These latter cells are able to inhibit T-cell proliferation, at least in part via production of nitric oxide, but do not induce T-cell apoptosis. Furthermore, adoptive transfer of the induced CD11b+ cells is sufficient to inhibit anti-CD40 therapy in tumor-bearing recipients. We have shown that the timing of cyclophosphamide relative to mAb administration is critical to the therapeutic outcome, and although the combination can improve survival, cyclophosphamide given prior to immunotherapy may generate a population of myeloid cells that can interfere with CTL responses and compromise the therapeutic outcome. (Cancer Res 2005; 65(16): 7493-501)

Introduction
Immunotherapy is becoming an established part of the treatment of cancer and a number of monoclonal antibodies (mAbs) are now in routine clinical use (1–3). The most promising results have been seen by combining mAb with chemotherapy or radiotherapy (4–7). These impressive results have led to chemoimmunotherapy becoming widely adopted in the treatment of hematological malignancies and certain solid cancers. However, little is known regarding the timing of mAb relative to chemotherapy, which is currently done empirically.

The DNA–alkylating agent cyclophosphamide is a key component of many chemotherapy regimens and is known to have direct antiproliferative and apoptotic effects (8). It also displays a range of contrasting immunomodulatory properties which vary mainly according to the treatment dose. Cyclophosphamide has been shown to augment delayed-type hypersensitivity reactions and has been employed in a variety of vaccination and adoptive immunotherapy protocols to potentiate antitumor immune responses (9–11). In contrast, high doses of cyclophosphamide have been used for many years as a component of immunosuppressing conditioning regimens prior to bone marrow transplantation as well as to suppress certain autoimmune disorders (12).

Most therapeutically active mAb seem to interact with either key signaling receptors, on the tumor itself, or on cellular effectors of the immune system (13). One such promising target is the CD40 antigen, which is expressed in a range of cell types including B cells and antigen-presenting cells, where it has an essential role in activating antigen-presenting cells to prime CTL responses (14). Treatment of a number of CD40+ or CD40− lymphomas with anti-CD40 mAb results in eradication of disease and induction of long-term immunity via the generation of a potent CTL response (15, 16). This activity can be further enhanced by combining the mAb with conventional strategies such as irradiation (17).

We reasoned that the capacity of cyclophosphamide to either activate or suppress the immune response was likely to have an important impact in combining cyclophosphamide-containing chemotherapy regimens with immunotherapeutic mAb. Here we have examined the efficacy of combining cyclophosphamide with anti-CD40 mAb for the treatment of B cell lymphoma and have shown that the timing of cyclophosphamide relative to mAb is critical to the long-term clearance of tumor. Pretreatment with chemotherapy inhibits the antitumor activity of the anti-CD40 mAb due to the emergence of a population of CD11b+ Gr-1+ cells that can suppress CTL proliferation, partly through production of nitric oxide (NO). Furthermore, this population of cells can inhibit anti-CD40 mAb-based therapy on adoptive transfer into naïve tumor-bearing recipients. This data provides new insights into the importance of the scheduling of chemotherapy relative to mAb in clinical trial design.

Materials and Methods

Animals and cell lines. BALB/c mice were supplied by Harlan UK (Blackthorn, United Kingdom). BCL1, is a syngeneic B-lymphoma line that develops primarily in the spleen, and was maintained by in vivo passage in BALB/c mice (18). The πBCL1 variant can be maintained both in vivo and in liquid culture (19).

All cell cultures were maintained in supplemented DMEM (Life Technologies, Paisley, United Kingdom) containing glutamine (2 mmol/L), pyruvate (1 mmol/L), penicillin and streptomycin (100 IU/mL), and 5% to

Requests for reprints: Timothy M. Illidge, CRUK Paterson Cancer Institute and Christie NHS Trust, Wilmslow Road, Manchester, M20 4BX, United Kingdom. Phone: 44-161-446-895, Fax: 44-161-446-8111; E-mail: tmi@manchester.ac.uk.

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10% FCS (Myocline, Life Technologies), except nBCL1, which was maintained in supplemented RPMI (Life Technologies) plus 50 μmol/L 2-mercaptoethanol (BDH, Poole, United Kingdom) and 10% FCS.

**Antibodies.** Antibodies used in this study were 3/23 (anti-CD40; ref. 20), Mc39-16 (anti-CD19A lymphoma idiotype; irrelevant control; ref. 13), KT3 (anti-CD3; ref. 21), 16-10A1 (anti-CD7) and Gr-1 (anti-CD7; both from American Type Culture Collection, Manassas, VA), anti-CD31 (Serotec, United Kingdom), anti-Gr-1/Ly-6G (BD Pharmingen, Oxford, United Kingdom), anti-CD11b (Serotec), and YTS 169 (anti-CD8; ref. 22). Hybridoma cells were maintained in stationary culture (as above) and prepared as previously described (13).

**Flow cytometry.** Mouse splenocytes were analyzed by direct immunofluorescence staining using a FACSCalibur (Becton Dickinson, Mountain View, CA). Briefly, splenocytes at 10^6/mL (100 μL sample) were incubated with FITC- or phycoerythrin-labeled mAb (50 μg/mL) for 5 minutes at room temperature, then washed with PBS containing 1% bovine serum albumin, and analyzed immediately.

**Immunotherapy.** Groups of age- and sex-matched mice were injected i.v. with tumor cells (10^6 BCL1 day 0) and were treated with anti-CD40 mAb i.v. as described in the text. Cyclophosphamide (200 mg/kg i.p.) or doxorubicin (6 mg/kg i.p.) was given at various time points relative to the mAb as described in the text. The tumor develops primarily in the spleen, with a terminal leukemic overspill. Survival was monitored daily, and the results were analyzed using the χ^2 test of Peto (23). Animal immunotherapy was approved by the local ethical committee of Southampton University and was done under a United Kingdom Home Office license.

**In vivo tracking.** To measure the kinetics of CTL expansion following treatment, groups of mice were inoculated with 10^6 lymphoma cells i.v. on day 0 and treated with cyclophosphamide or doxorubicin together with mAb as before. Animals were culled at sequential time points after treatment, spleens removed, homogenized, washed once by centrifugation, and resuspended at 10^6/mL in supplemented RPMI. CD8 T cells were detected by dual-fluorescence staining using phycoerythrin-YTS169 (rat anti-mouse CD8; ref. 22) versus FITC-KT3 (rat anti-mouse CD3).

**In vitro T cell proliferation.** CD11b+ cells were harvested from the spleens of tumor-bearing mice that had been treated with cyclophosphamide (200 mg/kg i.p., day 7) and anti-CD40 (500 μg i.v., day 15). Five days after the final treatment, CD11b+ cells were enriched using negative selection by MACS (Miltenyi-Biotec, Bergisch Gladbach, Germany) with anti-CD3-FITC and anti-CD19 phycoerythrin-labeled mAb, according to the manufacturer’s instructions. For the assay, culture plates (96-well U-bottom plates [Costar, Cambridge, MA]) were precoated with anti-CD3 mAb for 2 hours at 37°C (2 μg/mL). Splenocytes from BALB/c mice (0.5 × 10^6/well) were then added in a volume of 100 μL, together with CD11b+ cells at various effector/target (E/T) ratios (in a volume of 100 μL/well). Cells were incubated for 72 hours at 37°C in a CO2 incubator and T cell proliferation determined by the addition of 3[H]thymidine (0.5 μCi/well; Amersham International, Little Chalfont, United Kingdom) for the last 16 hours. Samples were harvested and counted on a TopCount Microplate Scintillation Counter (Packard, Meriden, CT).

For CD8+ T-cell proliferation, BALB/c splenocytes were first labeled with 5 μmol/L 5- (and 6-)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Leiden, the Netherlands) as described (13). Target splenocytes were then cultured as above. After 48 hours, samples were harvested, stained with phycoerythrin-labeled anti-CD8 mAb for 5 minutes at room temperature, washed and analyzed by flow cytometry. CD8+ T cell proliferation was determined by CFSE dilution, following gating on the CFSE−/CD8+ cell population. Typically, samples were run in triplicate and pooled for analysis.

Where indicated, the inhibitor of NO synthesis, 0.1 mmol/L Nω-monomethyl-L-arginine monooctate (n-NMMA; Alexis, San Diego, CA), was added to cultures.

**Measurement of nitric oxide.** Splenocytes were cocultured with anti-CD3 mAb and CD11b+ cells from tumor-bearing cyclophosphamide/anti-CD40-treated mice as for the standard T cell proliferation assay. After 48 hours of incubation, the levels of NO were measured as nitrite accumulation in the supernatant and determined using Greiss reagent (24).

**T cell apoptosis.** Apoptosis was determined using Annexin V-FITC (BD Pharmingen) as detailed by Vermes et al. (25). Briefly, culture plates (24-well multilipples; Life Technologies) were coated with anti-CD3 mAb (2 μg/mL) for 2 hours at 37°C prior to addition of cells (BALB/c splenocytes; 5 × 10^6/mL). CD11b+ cells, induced and prepared as before, were added at E/T ratios described in the text. Samples were harvested after 72 hours, washed once, and analyzed by flow cytometry. The percentage of Annexin V-positive cells was scored as apoptotic. As a positive control, dexamethasone (Sigma-Aldrich, United Kingdom) was added at a concentration of 0.1 μmol/L for the final 20 hours.

**Adoptive transfer therapy.** Mice were inoculated with 10^6 BCL1 tumor cells i.v. on day 0 and treated with anti-CD40 mAb (500 μg i.v.) on day 15. For adoptive transfer, mice received 5 × 10^7 splenic CD11b+ cells (purified from mice as before) intrasplenically 4 hours later. Controls received mAb alone or were given mAb plus 5 × 10^7 total splenocytes from untreated BALB/c mice.

**Results**

**Timing of cyclophosphamide relative to anti-CD40 mAb is critical to successful tumor protection.** Our initial experiments were aimed at exploring whether an additive therapeutic effect existed between cyclophosphamide and anti-CD40 mAb and whether the timing of cyclophosphamide relative to mAb was important to tumor protection. Previous work had shown that anti-CD40 mAb resulted in long-term clearance of tumor when given in relatively large or repeated doses (15). Therefore, we used smaller, single doses of anti-CD40 to examine whether a potentially additive effect could be observed with cyclophosphamide. Mice were inoculated with BCL1 lymphoma (10^6 cells, i.v.) on day 0 and were treated with either 1 mg of anti-CD40 mAb, 200 mg/kg cyclophosphamide, or a combination of both treatments as described. Figure 1A shows that treatment with a single dose of cyclophosphamide on day 7 results in around 25 days increased survival (median survival time of 51 days versus 25 days for control mice); treatment with anti-CD40 alone on day 15 provided long-term tumor-free survival (>100 days) to 60% of animals. Interestingly, for cohorts given combination therapy, pretreating with cyclophosphamide (day 7) followed by anti-CD40 (day 15), the degree of tumor protection was reduced to levels comparable to cyclophosphamide alone (median survival time 55 days). This apparent inhibitory effect of cyclophosphamide on anti-CD40 therapy was lost when the schedule was reversed and the mAb delivered prior to chemotherapy (i.e., anti-CD40 day 7, cyclophosphamide day 15). In these experiments, cyclophosphamide alone provided similar levels of protection as before, but the combination treatment now resulted in 80% long-term survival (Fig. 1B). When the dose of mAb was dropped to 100 μg, then a significant additive effect between the cyclophosphamide and mAb was observed under these conditions (P < 0.01; Fig. 1C). These results suggest that although an additive therapeutic effect could be shown between anti-CD40 and cyclophosphamide, this was critically dependent on timing, as pretreatment with cyclophosphamide actively inhibited the anti-CD40 mAb–mediated component of the therapy.

**Reduced CTL expansion and increased CD11b+ cell numbers after combination therapy.** We and others have shown that anti-CD40 therapy can induce tumor immunity through the generation of a potent CTL response (15–17). We thus looked to see if this response was affected following cyclophosphamide treatment, using in vivo tracking experiments. Mice treated with anti-CD40 mAb show a rapid increase in CTL 4 to 5 days posttreatment.
compared with controls (Fig. 2A), which coincides with a reduction in tumor volume (data not shown). Mice given cyclophosphamide alone show slightly reduced numbers of CTL compared with controls (1.5 × 10⁷ versus 2.5 × 10⁷, respectively), in-keeping with the lymphocytopenia to be expected with cyclophosphamide. Cohorts given cyclophosphamide plus anti-CD40 mAb generate levels of CTL similar to those in cyclophosphamide-treated animals (1.5 × 10⁷), indicating that the anti-CD40-facilitated expansion of CTL is inhibited by pretreatment with cyclophosphamide.

Interestingly, although there is a marked decrease in the number of CTL in the spleens of mice treated with the combination of anti-CD40 plus cyclophosphamide compared with controls, there was a highly significant increase in the number of CD11b⁺ cells present (Fig. 2B). Spleens harvested from tumor-bearing mice given no treatment or anti-CD40 alone contained ~6 × 10⁷ CD11b⁺ cells. Treatment with cyclophosphamide resulted in a 5-fold increase in the number of CD11b⁺ cells recovered (to ~3 × 10⁸/spleen). Numbers were further increased in mice that received combination treatment, to >6 × 10⁸ CD11b⁺ cells per spleen, a 10-fold expansion compared with controls. Similar increases in the number of CD11b⁺ cells were observed in non–tumor-bearing mice treated with cyclophosphamide or cyclophosphamide plus anti-CD40 compared with controls or those given anti-CD40 mAb alone. This data suggests that the emergence of this population was independent of the presence of tumor, related specifically to the administration of cyclophosphamide, and enhanced by the coadministration of anti-CD40 mAb (see Table 1).

There were also distinct phenotypic differences. CD11b⁺ cells from naïve mice, or those given cyclophosphamide alone, showed surface expression of Gr-1/Ly-6G (Fig. 2C) together with low levels of MHC I, LFA-1, FcγRII/III and CD11c (data not shown), but lacked B7.1, B7.2, and CD31 expression (Fig. 2C) as well as MHC II, ICAM-1, CD3, CD4, CD8, CD40, DEC-205, and DX5 (data not shown). However, surface expression of B7.1, B7.2, and CD31 was detectable on CD11b⁺ cells from animals given combination therapy (Fig. 2C), as well as a slight increase in ICAM-1 (data not shown), indicative of a more activated phenotype.

Cyclophosphamide induced CD11b⁺ cells inhibit T cell proliferation via production of nitric oxide. CD11b⁺ cells have previously been reported to reduce the ability of murine splenocytes to proliferate in response to mitogenic stimuli (26). Therefore, we hypothesized that cyclophosphamide-induced CD11b⁺ cells from our anti-CD40-treated lymphoma-bearing mice may also actively inhibit CTL proliferation. To examine this hypothesis further, CD11b⁺ cells were isolated from the spleens of therapy mice using MACS and their potential to inhibit T cell proliferation assessed in vitro. Coculture of anti-CD3 stimulated murine splenocytes with CD11b⁺ cells resulted in a dramatic reduction in proliferation (Fig. 3A). At E/T ratios as low as 0.1:1, a 40% decrease in proliferation could be observed, rising to >90% at a ratio of 2:1. At ratios higher than this (5:1, 10:1) virtually complete inhibition of T cell proliferation was observed (data not shown). Culture supernatant taken from mixed populations of anti-CD3-stimulated BALB/c splenocytes and CD11b⁺ cells, contained
detectable amounts of NO (up to 40 μmol/L), which was not present in unstimulated cultures (data not shown) or stimulated splenocytes in the absence of CD11b+ cells (Fig. 3B). Inclusion of the NO inhibitor L-NMMA was able to partially overcome the growth-inhibitory effects of the CD11b+ cells. Here, the proliferative response was increased by ~4-fold at the 2:1 E/T ratio and fully restored at lower ratios (Fig. 3A).

To specifically analyze the effect of CD11b+ cells on CD8+ T cell proliferation, a CFSE-based assay was used. Coculture of CFSE-labeled BALB/c splenocytes with CD11b+ cells was done as above. Proliferation was assessed after 48 hours and determined by dilution of dye in proliferating cells following dual staining with an anti-CD8 mAb. Inclusion of CD11b+ cells dramatically reduced CD8+ T cell proliferation in response to anti-CD3 mAb, such that <5% of the CD8+ T cells divided at the highest E/T ratio (2:1; Fig. 3C and D). The inclusion of L-NMMA again partly restored proliferation at the highest E/T ratio (up to 40% division) and, at the lower ratios, fully restored it to levels comparable with controls (Fig. 3D). At the concentrations used, the inhibitor had no significant effect on proliferation of control cultures.

The inhibitory effects on T cells were mirrored by CD11b+ cells taken from non–tumor-bearing animals treated with cyclophosphamide or cyclophosphamide plus anti-CD40. However, as for those cells isolated from tumor-bearing animals, the level of inhibition and NO production was greater for CD11b+ cells harvested from cyclophosphamide plus anti-CD40 treated animals compared with those from animals given cyclophosphamide alone, indicating that the mAb somehow exacerbated the suppressive properties of these cells (Table 1).

**CD11b+ cells do not induce CTL apoptosis.** We next wished to clarify whether the antiproliferative effect of the CD11b+ cells seen on the CD8 T cell population was due to growth arrest or whether these cells were inducing T cell apoptosis. We therefore measured surface phosphatidylserine expression on CTL following coculture with CD11b+ cells. CTL were analyzed by gating on CD8+ cells and apoptosis detected using FITC-labeled Annexin V mAb. As a positive control, splenocytes were incubated with dexamethasone (0.1 μmol/L), which induced ~53% apoptosis (shown in Fig. 4) with high levels of Annexin V staining. In contrast, lower levels of Annexin V staining were observed on CTL cultured with anti-CD3 alone (16.5%) or with CD11b+ cells at effector/target ratios of 2:1 (14.8%). Furthermore, these CTL remained negative for the viability stain 7-AAD, indicating that they retained membrane integrity (data not shown). This data indicates that CD11b+ cells do not function by inducing T cell apoptosis, suggesting that the observed reduction in T cell proliferation is a result of direct growth inhibition.

**Doxorubicin does not induce an increased population of CD11b+ cells and does inhibit anti-CD40 therapy.** To further investigate the specificity of this inhibitory response, we examined

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**Figure 2.** Reduced CTL expansion and increased numbers of activated CD11b+ cells in combination-treated mice. Mice received BCL1 (10^6 cells i.v., day 0) and were treated with cyclophosphamide (200 mg/kg i.p., day 7), anti-CD40 (500 μg i.v., day 15), or a combination of both. Animals were sacrificed 5 days after final treatment, spleens removed, and the number of CTL (A) and CD11b+ (B) macrophage/myeloid cells determined by flow cytometry using FITC-labeled anti-CD3 versus phycoerythrin-labeled anti-CD8, and phycoerythrin-labeled CD11b mAb, respectively. Columns, means; bars, SE. C, the phenotype of CD11b+ cells from cyclophosphamide or cyclophosphamide plus anti-CD40 mAb–treated animals was then assessed by two-color flow cytometry using phycoerythrin-labeled CD11b versus FITC-labeled irrelevant control (anti-A31 idiotype-binding mAb), anti-B7.1, anti-B7.2, anti-CD31, and anti-Gr 1 mAb. Combination therapy seems to inhibit CTL expansion in tumor-bearing animals, but leads to significantly increased numbers of activated CD11b+ cells present in the spleen compared with controls, or mice given cyclophosphamide alone (P < 0.01).
the effects of doxorubicin, another commonly used cytotoxic drug, on anti-CD40 therapy. Mice were inoculated with tumor and treated with either cyclophosphamide or doxorubicin (day 7) together with anti-CD40 (day 15). Animals were sacrificed 5 days later and the number of CD11b+ cells present in the spleen assessed. As shown in Fig. 5A, doxorubicin plus anti-CD40 stimulates only a modest increase in CD11b+ cell numbers compared with controls (~2-fold increase), whereas cyclophosphamide plus mAb results in a massive expansion (~10-fold over controls). Treatment with doxorubicin alone (6 mg/kg, i.p., day 7) extended survival by around 10 to 12 days (Fig. 5B). However, in contrast to cyclophosphamide, when doxorubicin was given in combination with anti-CD40, the level of protection observed was similar to that achieved with mAb alone, giving long-term protection to >80% of animals. Thus, it seems that chemotherapeutic reagents that do not stimulate increases in splenic CD11b+ cell populations do not inhibit anti-CD40 therapy.

Adoptive transfer of CD11b+ cells inhibits immunotherapy in naïve mice. To confirm that the cyclophosphamide-induced CD11b+ population was responsible for inhibiting anti-CD40-based

Table 1. T cell inhibition by CD11b+ cells. CD11b+ cells were isolated from the spleens of naïve or tumor-bearing mice treated with cyclophosphamide or cyclophosphamide plus anti-CD40 according to the standard protocol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Splenocyte proliferation (% of control)</th>
<th>NO production (μmol/L)</th>
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<tr>
<td></td>
<td>1:1*</td>
<td>0.1:1</td>
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<tr>
<td>Cyclophosphamide</td>
<td>36.64 ± 0.9</td>
<td>106.92 ± 6.4</td>
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<tr>
<td>Cyclophosphamide + anti-CD40</td>
<td>9.98 ± 2.3</td>
<td>45.99 ± 12.6</td>
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<tr>
<td>BCL1 + cyclophosphamide</td>
<td>45.48 ± 6.5</td>
<td>105.80 ± 6.1</td>
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<tr>
<td>BCL1 + cyclophosphamide + anti-CD40</td>
<td>15.63 ± 5.8</td>
<td>58.12 ± 8.6</td>
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NOTE: The percentages of proliferation of splenocytes cultured in the presence of the CD11b+ cells, together with levels of NO present in culture supernatant, are shown.

*Cyclophosphamide, splenocyte E/T ratios.

Figure 3. CD11b+ cells from therapy animals inhibit T cell proliferation via NO production. A, to assess the effect of CD11b+ cells on T cell proliferation, BALB/c splenocytes were stimulated with anti-CD3 mAb (plate bound; 2 μg/mL) and cocultured with CD11b+ cells (at E/T ratios shown) purified from the spleens of tumor-bearing mice (5 days after treatment with cyclophosphamide and anti-CD40 as before), in the presence or absence of L-NMMA (0.1 mmol/L) for 72 hours. Cells were pulsed with [3H]thymidine for the last 16 hours of the assay. The DNA-incorporated radioactivity was then harvested and counted. Columns, means; bars, SE. B, nitrite production in the supernatant of cells cultured as above was determined using Greiss reagent. C, CD8+ T cell proliferation was assessed using splenocytes labeled with CFSE prior to culturing as above. After 48 hours, triplicate samples were pooled, stained with phycoerythrin-labeled anti-CD8 mAb and analyzed by flow cytometry. Proliferation was determined by CFSE dilution following gating on the CD8+ population. Shown are representative histograms (C) and calculated percentage of cells divided (D). CD11b+ cells from therapy animals inhibit T cell proliferation, which can be partially overcome by blocking NO.
immunotherapy, we did adoptive transfer studies. Animals were inoculated with BCL1 lymphoma and treated with anti-CD40 on day 15 as before. For adoptive transfer, CD11b+ cells purified from a parallel group of cyclophosphamide and anti-CD40-treated animals were delivered by intrasplenic injection (5 x 10^7/mouse) 4 hours after anti-CD40 treatment. Controls received 5 x 10^7 total splenocytes from naive, BALB/c mice. Figure 6 illustrates that although cohorts given anti-CD40 mAb alone remain tumor-free, those given anti-CD40 mAb plus CD11b+ cells developed tumor (median survival time of 32 days, P > 0.01). This was in contrast to control animals receiving the same number of total splenocytes from naive BALB/c mice, which showed no difference in survival compared with animals given anti-CD40 alone. CD11b+ cells purified from tumor-bearing mice given cyclophosphamide alone also showed some inhibitory effect on anti-CD40 therapy in recipient mice (Fig 6B). However, the degree of suppression was not as complete as for CD11b+ cells taken from donors treated with combination therapy, further suggesting that addition of anti-CD40 promotes the inhibitory properties of these cells. Thus, it seems that adoptively transferred CD11b+ cells are able to inhibit CTL responses in tumor-bearing mice receiving anti-CD40 immunotherapy.

Discussion

Incorporating mAb with chemotherapy is rapidly becoming the established approach for many cancer types (1, 4–7). We have shown that the therapeutic efficacy of combining cyclophosphamide with anti-CD40 mAb is critically dependent on the balance between cyclophosphamide-associated immunosuppression and anti-CD40 mAb-mediated immune activation, which can be manipulated according to the treatment schedule employed. Importantly, pretreatment with cyclophosphamide reduces the therapeutic activity of the mAb, whereas protection is maintained and even enhanced if cyclophosphamide is delivered after the mAb (Fig. 1). Our findings suggest that cyclophosphamide inhibition of anti-CD40 mAb therapy relates to the expansion of a CD11b+ myeloid cell population in the spleen. Combination therapy results in an increased number of phenotypically activated CD11b+ cells compared with cyclophosphamide alone, which are able to suppress T cell responses through production of NO. Moreover, adoptive transfer of this myeloid fraction is sufficient to inhibit anti-CD40 therapy in tumor-bearing recipients.

Cyclophosphamide is known to have a direct cytotoxic effect on both normal and neoplastic cells which may impact on the cellular immune system. Indeed, treatment with cyclophosphamide at the doses described here results in a transient lymphocytopenia with numbers of CD19, CD4, and CD8 lymphocytes reduced by >90%, 70%, and 55%, respectively, for up to 2 weeks posttreatment (data not shown). However, we believe that the impaired CTL response is not simply a consequence of such cellular toxicity. Firstly, we have previously shown that combination of anti-CD40 mAb with equally immunosuppressive doses of irradiation could lead to long-term protection against lymphoma. Here, potent antitumor CTL responses were induced despite similar levels of lymphocytopenia (17). Second, depletion of CD8 T cells is apparent just 1 day after cyclophosphamide, however, treatment with anti-CD40 mAb within 24 hours of cyclophosphamide results in similar or greater levels of tumor protection than those achieved with mAb alone (data not shown). This shows that any general cytotoxicity by cyclophosphamide is not inclined at least in the short-term to prevent CD40 mAb-mediated antitumor responses.

Our previous work has shown the importance of tumor antigen load in triggering CTL responses that result in long-term clearance of tumor (16, 17). It is possible that pretreatment with cyclophosphamide may inhibit anti-CD40 therapy as a consequence of direct tumor cytotoxicity by reducing the quantity of tumor antigen available for CTL priming. However, our results with the adoptive transfer of purified CD11b+ population of cells to tumor-bearing cyclophosphamide naive animals strongly suggests that it is the CD11b+ cells and not the reduction in tumor antigen.
load that inhibits anti-CD40 therapy. In addition, long-term protection with anti-CD40 mAb is achieved when used in combination with other reagents such as doxorubicin, which induces similar levels of tumor cell kill, but not an increase in CD11b+ cells. Therefore, it seems that it is the emergence of the CD11b+ myeloid-enriched cell population in the spleen 7 to 10 days after cyclophosphamide administration that is responsible for impeding the CTL response. Our data is consistent with previous reports in demonstrating the appearance of a heterogeneous population of immature myeloid/monocyte cells induced by tumor cells (27) or chemotherapy (26, 28), and which are capable of inhibiting a variety of lymphocyte responses (29–31) and natural killer cell activity (32). These cells may have an important role in immune tolerance in the tumor-bearing host, where they seem to function to negatively regulate T cell responses. This regulation has been shown to be dependent on both NO and IFN-γ production (33), although some reports indicate that the immune suppressive population may inhibit CD8+ T cell function in a contact-dependent manner through induction of apoptosis (34, 35). In our lymphoma model, neither tumor growth nor treatment with mAb alone are sufficient to induce the suppressor population in the host, but the number of CD11b+ cells is moderately increased following treatment with cyclophosphamide, and further expanded in animals given anti-CD40 mAb in addition to cyclophosphamide (Fig. 3). Interestingly, the suppressor cells isolated from combination-treated mice had a more activated phenotype compared with those from mice given cyclophosphamide alone, and seemed to inhibit CTL proliferation at least in part through NO production rather than apoptosis (Figs. 4 and 5). Interestingly, the level of T cell growth inhibition achieved with CD11b+ cells from combination therapy mice seems to be greater on a per cell basis than with CD11b+ cells from mice given cyclophosphamide alone (Table 1). These cells were also more potent at inhibiting anti-CD40 therapy in recipient mice following adoptive transfer (Fig. 6). The observation that combination therapy should result in an increased number of suppressor-like cells compared with cyclophosphamide alone is intriguing. CD11b+ cell expansion and functional activity was greatest in cohorts treated with both cyclophosphamide and anti-CD40 irrespective of whether they were inoculated with tumor, suggesting that the emergence of this population was independent of tumor and was purely a consequence of combination treatment.

Figure 5. Doxorubicin does not induce an increased population of CD11b+ cells and does inhibit anti-CD40 therapy. A, mice received BCL1 (10⁶ cells i.v., day 0) and were treated with cyclophosphamide (200 mg/kg i.p., day 7) or doxorubicin (6 mg/kg i.p. day 7) together with anti-CD40 (500 µg i.v., day 15). Animals were sacrificed 5 days after final treatment, spleens removed, and the number of CD11b+ macrophage/myeloid cells determined by flow cytometry using phycoerythrin-labeled CD11b mAb. Columns, means; bars, SE. Animals were inoculated with tumor and treated with cyclophosphamide or doxorubicin plus anti-CD40 as above. Controls received PBS. Survival was monitored daily. Doxorubicin treatment in combination with anti-CD40 mAb does not lead to an increased number of splenic CD11b+ cells, and fails to inhibit the therapeutic properties of the mAb.

Figure 6. Adoptive transfer of CD11b+ cells can inhibit anti-CD40 therapy of naive animals. A, groups of five age- and sex-matched mice received BCL1 (10⁶ cells i.v., day 0) and were treated with anti-CD40 (500 µg i.v., day 15) together with 5 × 10⁷ control BALB/c splenocytes, or CD11b+ splenocytes (both given intrasplenically). Controls were total splenocytes prepared from spleens of naïve BALB/c mice. CD11b+ cells were purified from the spleens of tumor-bearing mice 5 days after treatment with cyclophosphamide and anti-CD40 mAb according to the standard protocol. B, as above, but donor CD11b+ splenocytes were purified from mice treated with cyclophosphamide alone. Survival was recorded daily. Mice that received anti-CD40 mAb plus adoptively transferred CD11b+ cells showed significantly reduced survival compared with mice given anti-CD40 plus control splenocytes (P < 0.01). One of at least two separate representative experiments is shown.
Treatment of mice with anti-CD40 alone did not result in an increase in CD11b+ cell numbers, although cells did show moderate up-regulation of B7.1, B7.2 and MHC II and low levels (<10 μmol/L) of NO production (data not shown). It is possible that these cells may have inhibitory properties too, but that the numbers present in the spleen are so low that they have no effect on therapy. Instead, it seems that anti-CD40 merely enhances the suppressor population induced by cyclophosphamide treatment. Quite why anti-CD40 should exacerbate the number and activity of these cells is intriguing. It has been shown that CD40 ligation may be an important trigger for NO production by early myeloid cells (36) as well as macrophages (37) and dendritic cells (38). Although we were not able to detect CD40 surface expression on the CD11b+ myeloid cell fraction induced in our tumor-bearing mice, it is still possible that anti-CD40 mAb could have a direct effect on these cells. It is also possible that local T cell activation triggered by anti-CD40 during the primary antitumor immune response serves to enhance the suppressive phenotype of these cells through IFN-γ release, which is known to be important for NO production by early myeloid cells (36). Thus, T cells activated by anti-CD40 may contribute to their own suppression through a “negative-feedback loop” involving further activation of the CD11b+ population via IFN-γ. However, this does not completely explain why the suppressor activity is increased in the absence of tumors. Alternatively, ligation of CD40 on tissue-resident cells, such as macrophages, may induce release of cytokines or other factors that promotes the activation and activity of these cells.

Interestingly, whereas cyclophosphamide has been shown to inhibit primary activation of naïve T cells, it can enhance the number of CD44hi CD8+ memory lymphocytes (39). This is believed to relate to bystander production of T cell growth and survival factors, noticeably type I IFN, released as a result of drug administration (39, 40). Some evidence suggests that there may also be a shift in the balance of the cytokine milieu from Th2 to Th1 (41). This observation may have some potential relevance to chemoimmunotherapy schedules using immunostimulatory mAb such as anti-CD40, which function through generation of CTL immunity. It is possible that a switch in the cellular environment favoring CTL activation and survival as a result of cyclophosphamide treatment may be schedule-dependent. Furthermore, it has recently been shown that metronomic dosing of cyclophosphamide can enhance antitumor CTL responses against a murine melanoma model (42). This strategy involves frequent administration of cyclophosphamide doses well below the maximum tolerated dose, resulting in reduced drug associated toxicity, and theoretically enabling immune function to be maintained. Although combination therapy led to enhanced responses over that observed with individual modalities, timing of cyclophosphamide treatment still seemed to have a pronounced effect on CTL numbers (42).

Interestingly, sensitivity seemed to correlate to different phases of the T cell response, such that recently activated effector T cells were more sensitive than those persisting cells displaying a memory phenotype. Whether metronomic dosing of cyclophosphamide in combination with anti-CD40 mAb may enhance tumor immunity over that seen with our current system and augment survival and persistence of memory T cells is an area for future investigation.

It is likely that different cytotoxic drugs will induce different levels of direct tumor cytotoxicity, or less expansion of the myeloid cell fraction, and it is important to evaluate alternative combinations. For example, we have observed that treatment with doxorubicin does not seem to induce the CD11b+ population in the BCL1 lymphoma model, and does not inhibit anti-CD40 mAb therapy in the same way that cyclophosphamide does (Fig. 5). Likewise, mAb targeting other antigens may operate through systems that remain unaffected by suppressor cells. Indeed, in certain circumstances, cyclophosphamide seems to enhance the antibody-dependent cellular cytotoxicity activity of some mAb (such as anti-MHC II), and the antiproliferative and apoptotic effects of others (such as anti-idiotypic mAb, data not shown). In conclusion, we have shown the importance of the sequencing of cyclophosphamide relative to immunotherapy. We believe that the scheduling of different drug and mAb combinations should be assessed carefully and immunologic end points considered in the design of chemoinmunotherapy sequencing. We suggest that this rational design approach is more likely to lead to improvements in tumor responses and to impact on cancer management in the future.

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Cyclophosphamide Inhibits Anti-CD40


Cyclophosphamide Inhibition of Anti-CD40 Monoclonal Antibody–Based Therapy of B Cell Lymphoma Is Dependent on CD11b + Cells

Jamie Honeychurch, Martin J. Glennie and Timothy M. Illidge