Synergistic Effect of Metronomic Dosing of Cyclophosphamide Combined with Specific Antitumor Immunotherapy in a Murine Melanoma Model

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ABSTRACT

Immunotherapy could be combined with conventional chemotherapeutic modalities aimed at reducing tumor burden. Such combination therapy may be most useful when "metronomic" doses of antineoplastic drugs are used, thereby potentially avoiding some of the immunosuppressive effects of these drugs. Recent studies have shown that some conventional antineoplastic drugs can be exploited for antiangiogenic capacities, a strategy that requires drugs to be administered at regular intervals. We therefore investigated whether such metronomic therapy with the alkylating agent cyclophosphamide (CTX) could be effectively combined with immunotherapy eliciting tumor-reactive CTLs. An immunization protocol using injection of recombinant DNA followed by injection of recombinant modified vaccinia virus Ankara strain was used to initiate a specific CTL response in mice capable of providing resistance to challenge with the murine melanoma B16.F10. Combining this immunotherapeutic regime with metronomic delivery of CTX resulted in antitumor activity that was dramatically enhanced over either treatment administered alone and was also significantly greater than combining immunotherapy with CTX administered by a maximum tolerated dose regime. Whereas both metronomic and maximum tolerated dose delivery of CTX did cause deletion of proliferating tumor-specific CTLs in the blood, this deletion occurred with slower kinetics with the metronomic schedule. Further analysis showed that metronomic CTX treatment did not delete cells with low expression of CD43, a "memory" phenotype, and that these cells maintained potent restimulatory capacity. The combination of immunotherapy and metronomic CTX therapy may be well suited to clinical management of cancer.

INTRODUCTION

CTLs directed at tumor cells presenting unique peptides on MHC class I molecules constitute a potentially powerful effector arm of host immunity to tumors. Immunization strategies that aim to induce or restimulate tumor-specific CTLs may therefore provide an effective and practical approach to cancer treatment (1). Whereas the utility of such immunotherapy has been shown in many preclinical studies, it is unlikely that this treatment modality alone will be sufficient to cure patients with significant metastatic disease and hence high tumor burdens. In fact, immunotherapy may hold most promise as an adjunct to conventional strategies aimed at cytoreduction, such as chemotherapy (2). However, it is well recognized that many chemotherapeutic drugs actively suppress cell-mediated immunity. CTX,[3] an alkylating agent commonly used in chemotherapy, is paradoxical in this regard. Whereas CTX does have immunosuppressive qualities and indeed is commonly used as a suppressant in autoimmune conditions such as arthritis and lupus nephritis, it has also been shown to have immune-

potentiating activity in some settings (3–7). Generally, these potentiating effects have been observed when the drug is administered before antigen exposure or, importantly, in low doses (8, 9).

It has recently been reported that proliferating endothelial cells forming new blood vessels within tumors are sensitive to the cytotoxic effects of many antineoplastic agents. More significantly, it was recently shown that this antiangiogenic capacity can be sustained when these agents are administered at frequent intervals, a so-called "metronomic" schedule (10, 11). Metronomic delivery was suggested to be improved over traditional chemotherapeutic regimes involving MTD because the latter require extended rest periods to allow the patient to recover from associated toxicity, a respite that also provides time for the endothelial compartment within tumor tissue to repair.

In general, the altered drug dosing used in metronomic dosing may be less toxic to normal tissues, and it is therefore possible that some degree of immune function could be maintained under this dosing regime. We therefore assessed the efficacy of antitumor responses generated by a combination of metronomic dosing of CTX with immunization strategies aimed at eliciting CTLs reactive to recombinant epitopes expressed by the murine melanoma model B16.F10. We show that the combination of metronomic administration of CTX with immunization provides antitumor responses that are dramatically enhanced over immunotherapy or chemotherapy alone and that are also enhanced over immunotherapy combined with CTX administered at the MTD.

MATERIALS AND METHODS

Mice. C57BL/6 mice were from breeding pairs originally obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained at the Biomedical Services Unit of John Radcliffe Hospital by brother × sister mating; *in vivo* experimental protocols were performed according to institutional guidelines.

In Vitro **Culture Media and Reagents.** All cultures were maintained in complete medium comprising RPMI 1640 (Sigma-Aldrich, Dorset, United Kingdom) with 2 mM glutamine, 1% penicillin-streptomycin, 5×10^{-5} M 2-mercaptoethanol (all from Invitrogen Ltd., Paisley, United Kingdom), and 10% fetal bovine serum (Globepharm, Guildford, United Kingdom).

Immunization Strategies. Animals were primed for influenza $NP_{366-374}$-specific CTL responses by i.m. injection of 50 μg of plasmid DNA encoding the *mel3* polyepitope (DNA-mel3). The *mel3* construct (12) consists of a string of five HLA-A2 and two HLA-A1 melanoma epitopes, and the influenza nucleoprotein epitope restricted by H-2D^b (ASNENMDAM). Only the $NP_{366-374}$ epitope is presented on a C57BL/6 background. Mice were boosted 14–60 days after DNA immunization by i.v. injection with 10^6 plaque-forming units of recombinant MVA encoding the *mel3* polyepitope construct (MVA-mel3).

Restimulation. In some instances, animals immunized against mel3 were boosted a second time by i.v injection with 1×10^6 syngeneic splenocytes that had been infected with recombinant vaccinia encoding the *mel3* polyepitope construct (Vacc-mel3). Splenocytes (5×10^7 cells/ml) were infected with 10^8 plaque-forming units of Vacc-mel3 in PBS supplemented with 0.1% BSA (Sigma-Aldrich) at 37°C for 2 h, washed, and injected i.v. into the lateral tail vein.

CTX Dosing Schedule. CTX (ASTA Medica Ltd., Cambridge, United Kingdom) was reconstituted in sterile distilled H_2O and administered by i.p. injection. The dosing schedules of Browder *et al.* (10) were adopted for these

studies. Thus metronomic dosing consisted of 175 mg/kg CTX injected every 6 days, and standard dosing consisted of a 21-day cycle of 150 mg/kg CTX administered every other day over 6 days (*i.e.*, three injections) followed by 15 days of rest.

Tumor Immunity Assay. One week after immunotherapy, groups of mice ($n = 5$) were challenged with B16.F10 melanoma cells (C57BL/6, H-2^b) that had been modified to express the *mel3* polyepitope construct in combination with enhanced GFP from a bicistronic mRNA (pIRES2-EGFP vector; Clontech, Basingstoke, United Kingdom; Ref. 13). Challenge was with 3×10^5 tumor cells injected s.c. into the left flank. Mice were monitored for tumor growth every 3–4 days, and tumor size for each group was calculated as the mean of the products of bisecting diameters ($\pm$SE.). Measurements were terminated for each group when the first animal developed a tumor in excess of 200 mm^2.

Monitoring CTL Responses with MHC Tetramers. Tetrameric H-2 D^b/NP$_{366-374}$ peptide complexes were prepared as outlined in Altman *et al.* (14) and used to stain fresh peripheral blood lymphocytes isolated from the lateral tail vein. Approximately 5×10^5 peripheral blood lymphocytes were suspended in 20 μl of complete medium and incubated with 0.5 μg of tetramer complexes at 37°C for 20 min. The cells were then incubated with anti-CD8α and anti-CD43 (BD PharMingen, San Diego, CA) for 10 min at 4°C, washed twice with PBS, and resuspended in PBS for FACS analysis. Cells were analyzed with FACScan hardware and CellQuest software (BD Biosciences, Mountain View, CA). Blood was also collected to perform WBC counts so that total lymphocyte numbers per mm^3 of blood could be extrapolated. WBC counts were performed using a Micros60 blood counter (ABX-PARC Euromedecine, Montpellier, France).

Statistical Analysis. The statistical significance of differential findings between experimental groups was determined by Student's *t* test. Findings were regarded as significant if two-tailed *P* values were <0.05.

RESULTS

Sequential Immunization of C57BL/6 Mice with DNA and Vaccinia Vectors Encoding a Tumor-Specific Antigen Can Elicit CTL Responses Capable of Resisting Tumor Challenge. A murine model of melanoma expressing a unique CTL epitope was used to assess specific immune responses to tumors *in vivo*. It has previously been shown that an immunization strategy involving sequential injection of recombinant DNA and vaccinia vectors encoding a specific epitope can elicit powerful CTL-mediated immune responses (15–17). Immunization with vectors encoding the polyepitope construct *mel3* can be used to initiate CTL responses in both H-2^b mice and HLA-A2-transgenic mice (12). In H-2^b strains, such as C57BL/6, CTL responses are induced solely to the D^b-restricted influenza nucleoprotein epitope (NP$_{366-374}$). We therefore assessed the efficacy of sequential immunization with DNA and vaccinia (MVA; Ref. 18) vectors encoding *mel3* to induce NP$_{366-374}$-specific responses capable of resisting challenge with B16 melanoma cells also modified to express *mel3*. DNA (DNA-mel3) was injected i.m. to prime NP$_{366-374}$-specific CTLs, and then these responses were boosted several days later by i.v. injection of recombinant MVA (MVA-mel3). Proliferation of specific CTLs was monitored in the blood using H-2 Db/NP$_{366-374}$ tetramers and FACS analysis. In a typical experiment shown in Fig. 1*A*, DNA-mel3 alone induced activation and proliferation of NP$_{366-374}$-specific CTLs such that by day 13 after injection, the tetramer-positive population represented an average of $1.5 \pm 0.21\%$ of circulating CD8$^+$ lymphocytes ($n = 5$). When MVA-mel3 was injected i.v. 14 days after DNA immunization, the NP$_{366-374}$-specific cells were effectively boosted, proliferating to an average of $25.6 \pm 4.5\%$ of CD8$^+$ lymphocytes 7 days later. For the tumor challenge experiment shown in Fig. 1*B*, B16-mel3 melanoma cells were injected s.c. 7 days after MVA-mel3 boost. These cells had been transfected with the *mel3* polyepitope construct together with a GFP sequence to monitor antigen expression. Antitumor responses were observed in all immunized animals, with engraftment and
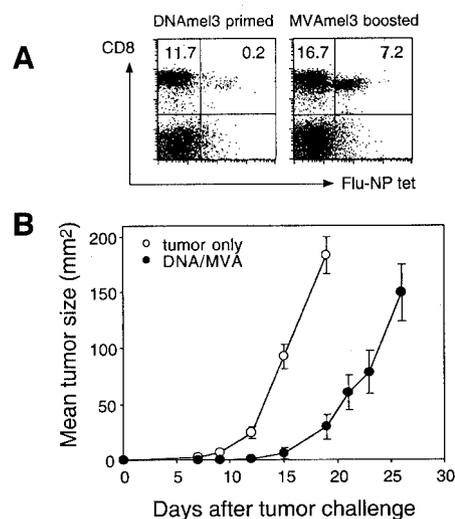

Fig. 1. Sequential immunization with recombinant vectors encoding a tumor-specific antigen induces resistance to tumor challenge. CTL responses in C57BL/6 mice were primed by i.m. injection of DNA-mel3 and then boosted by i.v. injection with MVA-mel3 14 days later. *A*, representative FACS plots showing expansion of a population of Flu-NP$_{366-374}$/D^b tetramer$^+$ CD8$^+$ cells in the blood in response to this immunization regime are presented. Percentages of cells in the upper quadrants are indicated. *B*, immunized animals were challenged with 3×10^5 B16-mel3 cells 7 days after MVA boost, and tumor progression was compared with growth observed in unimmunized animals. Mean tumor size per group ($n = 5$) $\pm$ SE is shown.

growth of the administered tumors retarded by 9 days relative to growth in control animals (Fig. 1*B*). Analysis of GFP expression in tumors that eventually developed in the immunized animals indicated selective loss of antigen expression (data not shown). These data indicate that this immunization strategy can induce stimulation of significant antitumor responses, but immunological escape occurs by antigen loss.

Metronomic Dosing with CTX Enhances Antitumor Effects of CTL-Mediated Immunity Induced with Recombinant Vectors. The immunotherapy model described above was used to investigate the feasibility of combining CTX treatment with antitumor immunotherapy (Fig. 2*A*). CTX treatments commenced 10 days after tumor challenge. CTX treatment alone, whether delivered as MTD or metronomic schedule, induced antitumor activity resulting in retarded tumor growth relative to untreated controls. Measurements were terminated for each group when the first animal developed a tumor in excess of 200 mm^2. Thus, whereas measurements were terminated for the untreated group at day 21 after challenge, animals undergoing the MTD regime survived until day 34 after tumor challenge, and animals receiving the metronomic schedule survived until day 42 after challenge. Significantly, the combination of immunotherapy and metronomic dosing of CTX was the most successful of all of the treatment regimes. The first animal in this group bearing a tumor in excess of 200 mm^2 was sacrificed at 81 days after challenge (*versus* 48 days in the immunotherapy/MTD group). Two of five animals were still tumor free at this point, and they remained tumor free in excess of 100 days after challenge in the absence of further CTX treatment, until they too succumbed to tumor. In contrast, all animals in the other groups were bearing tumors at the time measurements were terminated and animals were sacrificed. It is noteworthy that by day 40 after tumor challenge, when all of the CTX treatment groups had received approximately the same overall CTX dose regardless of administration schedule (875 mg/kg for the metronomic group; 900 mg/kg for the MTD group), the combined immunotherapy and metronomic CTX treatment group already had a significantly lower tumor burden than the other groups (mean tumor size for metronomic CTX
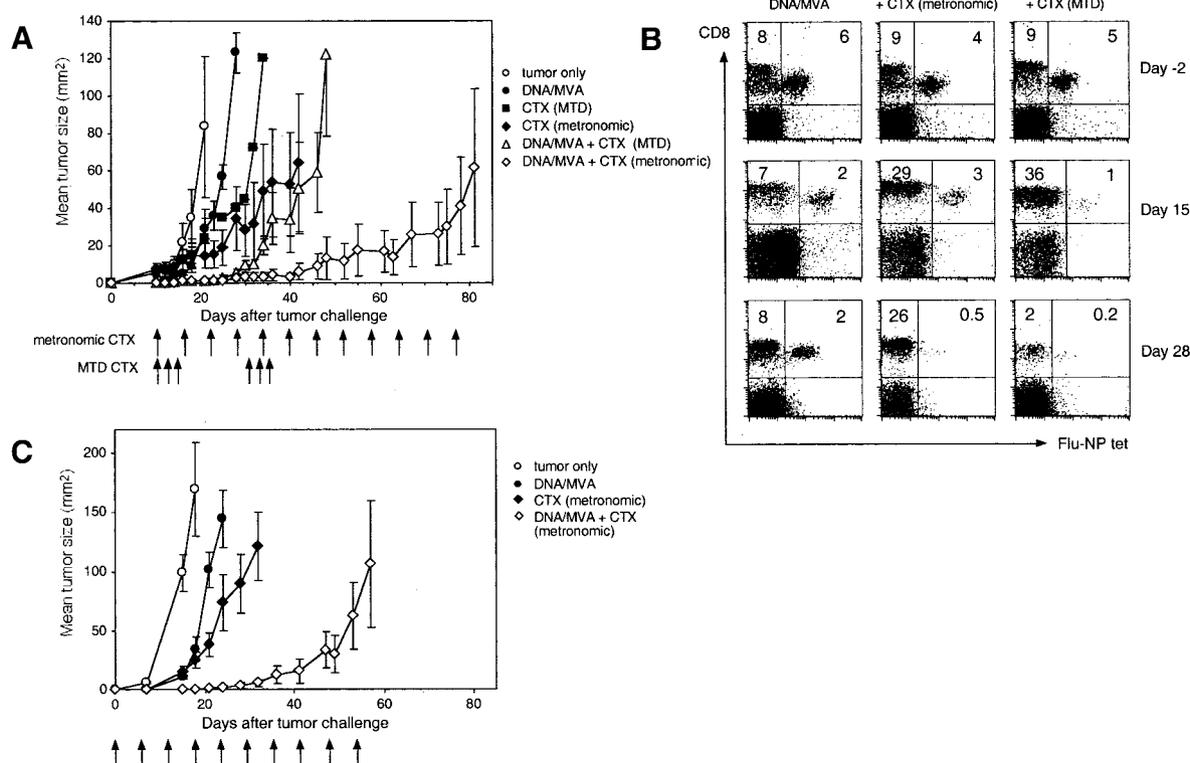
Fig. 2. Combining immunotherapy with metronomic dosing of CTX provides prolonged resistance to tumor challenge. *A,* progression of B16-mel3 tumors was monitored in animals that were subjected to one of the following treatment schedules: no treatment (○); immunization before challenge as outlined in the Fig. 1 legend (●); treatment with CTX administered as MTD (repeated cycles of 150 mg/kg every other day for 6 days followed by 15 days of rest; ■); metronomic treatment with CTX (175 mg/kg every 6 days; ◆); a combination of prior immunization and CTX by MTD (△); or a combination of prior immunization and metronomic CTX (◇). *Arrows* indicate CTX dosing; CTX treatments commenced on day 10 after tumor challenge. Mean tumor size per group ($n = 5$) $\pm$ SE is shown. *B,* blood was taken from immunized animals 2 days before tumor challenge and 15 and 28 days after challenge to examine the effects of CTX treatment on levels of Flu-NP$_{366-374}$-specific CTLs, as measured by FACS analysis with Flu-NP$_{366-374}$/D^b tetramers. Representative FACS plots are shown, and percentages of cells in the upper quadrants are indicated. *C,* a repeat of the experiment shown in *A* without groups treated with CTX by MTD, and with metronomic CTX treatment initiated on the day of tumor challenge.

and immunotherapy = 3 ± 2 mm^2, *versus* 34 ± 17 mm^2 for MTD CTX and immunotherapy). These data suggest a synergistic antitumor response when metronomic delivery of CTX was combined with immunotherapy.

Effects of CTX on Lymphocyte Numbers in Blood. Analysis of WBC counts from animals involved in the above experiment at day 40 after tumor challenge indicated that all of the groups treated with CTX underwent cytopenia, although there were no significant differences in cell counts between the drug treatment regimes (average for CTX-treated groups = 1.5×10^3 cells/mm^3 blood, *versus* 10.3×10^3 cells/mm^3 blood for control animals). However, FACS analysis of blood at different time points over the experiment indicated that there were differences in the kinetic profiles of lymphocytopenia induced by the different drug administration regimes (Fig. 2*B*; Table 1). Both

strategies resulted initially in a reduction of CD8$^-$ cells in preference to CD8$^+$ cells, as indicated by a higher proportion of CD8$^+$ cells at day 15 after challenge. However, significantly, the metronomic CTX treatment resulted in retention of a higher proportion of NP$_{366-374}$-specific lymphocytes in the blood ($2.8 \pm 0.4\%$) than that seen in animals receiving CTX at MTD ($0.9 \pm 0.1\%$), although for both treatment groups, the proportion of specific CTLs as a percentage of CD8$^+$ cells was considerably reduced relative to the immunized animals not receiving CTX. Thus, whereas the CTX treatment selectively reduced the numbers of tumor-specific CTLs, presumably by targeting proliferating cells, this effect was lower in animals undergoing metronomic treatment. This observation was most likely due to the lower overall dose of CTX that these animals had received by day 15 (a total of 175 mg/kg for the metronomic group *versus* 450 mg/kg for the

Table 1 *FACS analysis of groups of animals treated with immunotherapy and CTX[a]*

	CD8$^+$ Flu-NP tet$^+$	CD8$^+$ Flu-NP tet$^-$	CD8$^-$ Flu-NP tet$^-$
Day -2			
DNA/MVA	3.9 ± 1.1	10.7 ± 0.6	85.4 ± 1.5
DNA/MVA + CTX (metronomic)	3.1 ± 1.0	9.3 ± 1.2	87.6 ± 0.9
DNA/MVA + CTX (MTD)	3.3 ± 0.7	11.6 ± 1.3	85.1 ± 0.8
Day 15			
DNA/MVA	2.8 ± 0.4	8.4 ± 0.3	88.7 ± 0.7
DNA/MVA + CTX (metronomic)	2.8 ± 0.6	27.7 ± 1.6	69.5 ± 1.0
DNA/MVA + CTX (MTD)	0.9 ± 0.1	34.8 ± 1.6	64.2 ± 1.5
Day 28			
DNA/MVA	1.8 ± 0.5	11.1 ± 0.9	87.0 ± 1.0
DNA/MVA + CTX (metronomic)	0.7 ± 0.2	25.7 ± 1.8	73.6 ± 1.7
DNA/MVA + CTX (MTD)	0.2 ± 0.1	2.7 ± 0.4	97.1 ± 0.4

[a] Data were taken from groups of animals in the experiment in Fig. 2, *A* and *B*, showing mean percentage of lymphocytes with the indicated phenotype $\pm$ SE.
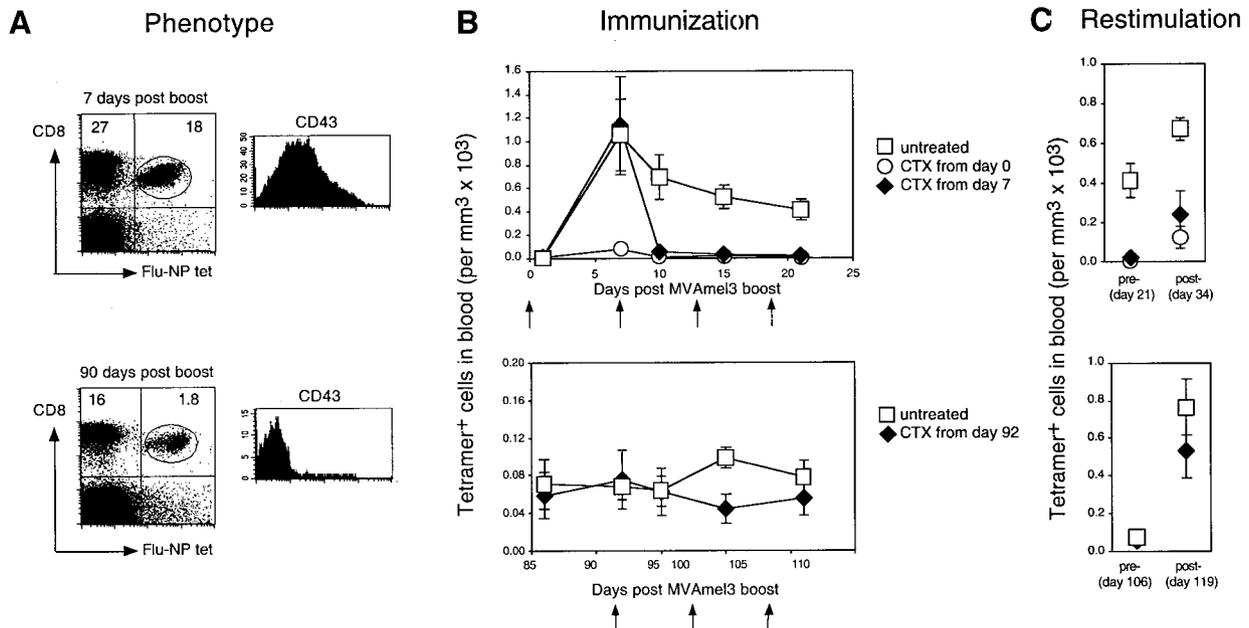
Fig. 3. Activated CTLs with a CD43 low phenotype are resistant to metronomic CTX-mediated deletion and retain potent restimulatory capacity. *A*, CTL responses were initiated by immunization as described in the Fig. 1 legend, and then blood was taken for FACS analysis on the indicated days. Percentages of cells in the upper quadrants are indicated. CD43 expression was assessed on gated CD8$^+$ Flu-NP$_{366-374}$/D^b tetramer$^+$ cells. *B*, CTL responses were monitored in the blood of immunized animals after metronomic CTX treatment initiated on the days indicated. The schedule of CTX administration is indicated with *arrows*. The total number of CD8$^+$ Flu-NP$_{366-374}$/D^b tetramer$^+$ cells/mm^3 blood was extrapolated from FACS data combined with lymphocyte counts determined using a Micro60 blood counter. Data are presented as mean number of CD8$^+$ Flu-NP$_{366-374}$/D^b tetramer$^+$ cells/treatment group ($n = 5$) $\pm$ SE. *C*, each of the treatment groups in *B* was injected with Vacc-mel3-infected syngeneic splenocytes to restimulate Flu-NP$_{366-374}$-specifc CTLs (day 21, *top panel*; day 106, *bottom panel*). The mean number of CD8$^+$ Flu-NP$_{366-374}$/D^b tetramer$^+$ cells in the blood per group before injection (*pre-*) and 7 days after injection (*post-*) is shown.

MTD group). Therefore, a significantly larger potentially tumor-reactive CTL pool was retained in the blood of animals receiving metronomic treatment in the first few days after treatment was commenced. By day 28, the number of NP$_{366-374}$-specific CD8$^+$ cells in both CTX-treated groups was at the lower level of detection. Notably, the higher doses of CTX resulted in a more profound reduction in all CD8$^+$ cells by this later period, which was not observed in the metronomic treatment group. These data suggest that by avoiding some of the cytotoxicity associated with the MTD schedule, the metronomic schedule permits a longer period of CTL activity and hence provides a more efficacious combination with immunotherapy.

Effects of Timing of CTX Therapy on Antitumor Response. For the above experiment, CTX treatment (by either dosing regime) was initiated 10 days after tumor challenge, which was 17 days after MVA boost. Interestingly, when metronomic therapy was initiated on the day of tumor challenge (Fig. 2C), the combination therapy was not as potent as in the earlier experiment, although the combination therapy was still greatly enhanced over the other treatment groups. In this experiment, the metronomic therapy had been initiated at a time when the CTL proliferation was likely to be peaking after MVA boost.[4] Thus, these results imply that the toxicity associated with CTX treatment, even at the metronomic doses used, still has detrimental activity on proliferating CTLs, thereby weakening the antitumor responses. Therefore, whereas the combination of immunotherapy and metronomic dosing of CTX provides a powerful treatment strategy, prudent timing of chemotherapy relative to immunotherapy may be required to provide the most effective response.

Metronomic Dosing with CTX Reduces Numbers of Proliferating "Effector" CTLs but Spares CTLs with Restimulatory Capacity. The effect of metronomic delivery of CTX on CTL proliferation and restimulatory capacity was studied in the context of

immunization with recombinant *mel3* vectors as outlined for the experiments above, but without subsequent tumor challenge. Two cohorts of animals were examined; one that had been recently immunized, and another that had been immunized 3 months previously. Phenotypic analysis indicated that CTLs in the recently immunized (7 days after boost) expressed high levels of CD43, a phenotype associated with CD8$^+$ T cell "effector phase" (Fig. 3A, *top panel*; Ref. 19). In contrast, analysis of CTLs in animals immunized 3 months previously showed low levels of CD43 expression, a phenotype that has been associated with CD8$^+$ T-cell memory (Fig. 3A, *bottom panel*).

CTX was administered to these animals by metronomic schedule at different times relative to MVA-mel3 boost to observe the effect of timing of CTX treatment on numbers of NP$_{366-374}$-specific cells detected in the blood. For these analyses, total numbers of NP$_{366-374}$-specific cells in the blood were extrapolated from WBCs counts, thereby taking into account the general reduction in numbers of WBCs observed in CTX-treated animals. The effect of CTX on NP$_{366-374}$-specific responses was found to be dependent on the timing of initiation of drug treatment (Fig. 3B, *top panel*). Thus, when CTX treatment was initiated on the same day as MVA boost, CTL proliferation was significantly impaired relative to immunized animals that did not receive CTX ($P = 0.043$, Student's *t* test), with total numbers of CTLs at day 7 after boost reduced by 90%. Delaying drug treatment an additional 7 days permitted initial CTL proliferation. However, these recently stimulated cells were also susceptible to CTX treatment, and numbers were rapidly reduced after drug treatment was initiated. Within 3 days of CTX treatment, the numbers of specific CTLs were reduced by 92% relative to immunized animals that did not receive CTX. In contrast, when CTX was given to animals that had been primed and boosted 3 months previously, the levels of NP$_{366-374}$-specific CTLs in the blood were not affected by drug treatment (Fig. 3B, *bottom panel*).

Potent *in vivo* re-stimulatory capacity is a hallmark of T-cell mem-

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[4] M. J. Palmowski, unpublished observations.

ory. To further investigate whether metronomic CTX treatment spares cells with a memory phenotype, each of the groups of animals above was subjected to a boosting regime involving i.v. injection with syngeneic splenocytes that had been infected *ex vivo* with the recombinant vaccinia virus Vacc-mel3. This strategy was used because the previous MVA infection would have generated antibodies capable of neutralizing a second vaccinia virus infection. By administering splenocytes that had been infected *ex vivo*, these neutralizing antibodies could be avoided temporarily, permitting presentation of the $NP_{366-374}$ peptide and hence allowing expansion of $NP_{366-374}$ CTLs. CTX treatment was terminated 6 days before injection of infected splenocytes. Increases in numbers of $NP_{366-374}$-specific CTLs after injection of mel3 vaccinia-infected splenocytes were observed in all animals, although this expansion was not statistically significant in animals that had received CTX during or soon after MVA boost (day 0 or day 7 after boost; Fig. 3C, *top panel*). In contrast, significant increases in $NP_{366-374}$-specific CTLs were observed in those animals that were boosted >90 days previously ($P = 0.036$; Fig. 3C, *bottom panel*), with boosting responses similar to those of control groups that were not treated with CTX ($P < 0.05$). These data demonstrate that CTX treatment causes a reduction in the numbers of proliferating CTLs but does spare a cohort of cells with restimulatory capacity.

DISCUSSION

Bolus administration of chemotherapeutic drugs at the MTD is known to be associated with considerable toxicity and discomfort to cancer patients. In light of recent studies describing the potent antiangiogenic capacity of drug administered in a metronomic fashion (10, 11), it is likely that metronomic dose regimes will find favor in the clinical setting. The study presented here highlights another important advantage of metronomic drug treatment, namely that the lowered associated toxicity permits a degree of immune function and hence the potential to combine drug treatment with immunotherapy. Specifically, we have shown that metronomic dosing of CTX combines very effectively with antitumor immunotherapy administered by injection of recombinant vectors encoding a tumor-specific antigen. These therapeutic combinations could potentially be applied to a variety of neoplasms for which tumor antigens have been defined.

The balance between CTX-induced cytotoxicity on the one hand and induction of effective immunity on the other is particularly fine. Whereas metronomic administration of CTX caused significant reduction in CTL numbers in the blood immediately after initiation of drug treatment, the combination therapy was still dramatically enhanced over either treatment alone. Significantly, the combination of metronomic CTX and prime boost-mediated immunity was more potent than combination therapy using MTD of the drug. This result highlights the importance of reducing CTX-induced cytotoxicity directed at the immune effector cells. Supporting this suggestion, FACS analysis indicated that more CTLs persist in the blood of animals receiving the metronomic treatment compared with animals receiving the MTD. Interestingly, where metronomic dosing was used on immunized animals without tumor challenge, the CTLs appear to succumb to the CTX more rapidly (compare Fig. 2B, *middle column*, CTX from day 10 and Fig. 3B, *top panel*, CTX from day 7). It is possible, therefore, that the presence of tumor may provide some CTL restimulation, thereby sustaining numbers in the blood for a longer period. Despite the detrimental effect of CTX on numbers of CTLs, the combined antitumor activity of CTLs and CTX was significant, implying that sufficient CTLs survive to have impact. It should be pointed out that for purposes of tracking immune responses over time, all CTL analyses were performed by monitoring the blood. Homing of lymphocytes in the presence of CTX was not addressed in these studies, and it remains possible therefore that redistribution of CTLs could account for some of the observed changes in CTL frequencies in the blood.

It is not yet clear whether the antiangiogenic environment created by the metronomic scheduling of CTX has any potentiating role with respect to CTL function. We are currently investigating this possibility using antiangiogenic agents that, unlike CTX, have no direct cytotoxic activity on the tumor cells. It is likely that the efficacy of the combination therapy is a result of efficient CTL-mediated cytolysis in the first instance, which effectively reduces the tumor burden on which the drug must act. Thus, whereas loss of antigen after immunotherapy alone results in unimpeded tumor growth, sustained CTX treatment is able to keep growth of the antigen-loss variants in check for a considerably enhanced period. Alternatively, the CTX treatment, whether by minimizing angiogenesis or by acting on the tumor cells directly, may be preferentially removing proliferating tumor cells. The remaining cells, with a slower proliferative capacity, may take more time to provide antigen-loss variants that can avoid CTL-mediated deletion. Interestingly, the $CD8^+$ fraction of lymphocytes seemed to be more resistant to the cytotoxic effects of CTX than the $CD8^-$ cells. This trend was more pronounced under the metronomic regime than the treatment with MTD. It is possible that this $CD8^+$ fraction contains CTLs specific to tumor-specific antigens other than $NP_{366-374}$ that contribute to the overall antitumor immune response, perhaps through epitope spreading. Additionally, the $CD8^-$ population prone to CTX-mediated cytolysis may include regulatory T cells such as the $CD4^+CD25^+$ population shown to suppress antitumor activity in other studies (20, 21). Indeed, removal of a suppressor population has been proposed as one of the mechanisms providing the previously reported immune potentiating effects of doses of CTX before immunotherapy (22). Yet another possible explanation for the synergy between metronomic CTX and specific antitumor immunotherapy could be drug-mediated increases in tumor immunogenicity (23, 24) or CTX-induced alterations within the tumor stroma (25).

The doses of CTX used in this study were taken directly from the report of Browder *et al.* (10), in which the antiangiogenic property of metronomic low-dose CTX therapy was first reported. These experiments were also performed on C57BL/6 mice, with the metronomic dose provided as approximately one-third of the MTD. It should be noted that some weight loss and discomfort were observed in animals that were subjected to metronomic dosing for periods in excess of 90 days. Thus it may still be desirable to lower the dose even further. A recent report has shown that CTX can be administered constantly in drinking water, effectively lowering CTX dose while sustaining antiangiogenic capacity (26). It is likely that such a strategy would result in less immune suppression and less associated morbidity.

The effect of CTX on numbers of CTLs in the blood appears to be dependent on the phenotype of these antigen-experienced cells. Whereas even the metronomic schedule induces a rapid reduction in numbers of recently activated cells, this reduction is avoided if drug administration is delayed for a period of months after MVA boost, by which time the majority of activated lymphocytes had reverted to a CD43 low phenotype. This phenotype has been described by Harrington *et al.* (19) to be a memory phenotype. Our experiments show that these cells retain a significant restimulatory capacity, a hallmark of memory, regardless of whether they had been exposed to CTX or not (Fig. 3C). CTX is a well-recognized alkylating agent, with its activity directed at the DNA of dividing cells. It is not surprising, therefore, that CTLs in a proliferating effector phase fall prone to the activity of this drug, whereas cells activated months earlier, and approaching quiescence, do not.

It should be possible to exploit this knowledge in the design of combination treatment schedules. Where primary treatment either

through surgical extirpation or radiation therapy has not been curative, or where the risks of developing subsequent metastatic disease are high, combination treatment could be administered in an adjuvant setting. Immunotherapy could be initiated well before metronomic dose CTX administration to minimize immunosuppression. With the first signs of recurrent disease, the tumor-specific memory compartment can be effectively boosted by immunotherapy during a temporary respite from metronomic dosing. Alternatively, in the setting of advanced local or metastatic disease, MTD chemotherapy could be initiated at sufficiently high doses to debulk the tumor, followed by combination therapy as described. Such combined treatment approaches, instituted with judicious timing, could well be appropriate for a disease as chronic and complex as cancer.

ACKNOWLEDGMENTS

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