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Chronic Chemoimmunotherapy Achieves Cure of Spontaneous Murine Mammary Tumors via Persistent Blockade of Posttherapy Counter-Regulation

Rachael B. Rowswell-Turner, Jamie L. Harden, Raji E. Nair, Tao Gu, Mehmet O. Kilinc, and Nejat K. Egilmez

Intratumoral delivery of IL-12 and GM-CSF induces local and systemic antitumor CD8⁺ T cell activation and tumor kill. However, the effector response is transient and is rapidly countered by CD4⁺ Foxp3⁺ T suppressor cell expansion. To determine whether depletion of the pre-existing T suppressor cell pool prior to treatment could diminish posttherapy regulatory cell resurgence, FVBneuN mice bearing advanced spontaneous mammary tumors were treated with cyclophosphamide (CY) 1 d before IL-12/GM-CSF therapy. Administration of CY mediated a significant delay in the post–IL-12/GM-CSF T suppressor cell rebound, resulting in a 7-fold increase in the CD8⁺ CTL/T suppressor cell ratio, a 3-fold enhancement of CTL cytotoxicity, and an extension of the effector window from 3 to 7 d. In long-term therapy studies, chronic chemoimmunotherapy promoted a dramatic enhancement of tumor regression, resulting in complete cure in 44% of the mice receiving CY plus IL-12/GM-CSF. Tumor eradication in the chronic therapy setting was associated with the ability to repeatedly rescue and maintain cytotoxic CD8⁺ T cell activity. These findings demonstrated that chronic administration of CY in conjunction with immune therapy enhances the initial induction of antitumor T effector cells and, more importantly, sustains their cytotoxic activity over the long-term via persistent blockade of homeostatic counter-regulation. The Journal of Immunology, 2011, 187: 4109–4118.

S ustained delivery of IL-12 and GM-CSF to the tumor microenvironment restores cytotoxicity to tumor-resident CD8⁺ T effector/memory cells, eliminates pre-existing CD4⁺ CD25⁺ Foxp3⁺ T suppressor cells, and promotes the priming of a secondary CTL response in the tumor-draining lymph nodes (TDLN) (1). However, T effector cell activity is transient and is rapidly followed by a T suppressor cell rebound, resulting in premature termination of antitumor cytotoxic activity (2, 3). Repeated stimulation can rescue cytotoxic activity, yet counter-regulation intensifies with each treatment, ultimately resulting in complete loss of the therapeutic effect (2). The phenomenon of treatment-induced feedback inhibition is distinct from tumor-mediated immune suppression and represents a robust and persistent barrier to obtaining durable effector responses. Although the role of T cell-intrinsic negative-checkpoint molecules, such as CTLA-4 and programmed death 1, in the regulation of T effector cell activity has been studied extensively (4, 5), the biology of poststimulation regulatory cell resurgence is less-well characterized, particularly in the context of cancer immune therapy. CD4⁺ CD25⁺ Foxp3⁺ T suppressor cells either develop in the thymus (central) or are induced in the periphery (peripheral) from naive precursors (6). In tumors, both populations contribute to immune suppression, but pre-existing central T suppressors have been identified as the predominant source of posttherapy regulatory cell expansion (7, 8). Therefore, we hypothesized that depletion of the tumor/TDLN-resident T suppressor pool prior to IL-12/GM-CSF therapy could diminish treatment-induced regulatory rebound. Of the numerous T suppressor cell-depletion strategies that have been tested, use of chemotherapeutics stands out because of its efficacy and simplicity of use (9, 10). Specifically, the ability of cytotoxic drugs to augment antitumor immune responses, a phenomenon that was reported >4 decades ago (11), was shown to involve several distinct mechanisms, including the enhancement of tumor cell immunogenicity (10, 12), the preferential depletion of regulatory cells (9, 10, 13), and the induction of a homeostatic recovery environment that is rich in growth-promoting cytokines (14). Although the mechanisms underlying the synergistic activity of select chemotherapeutics on T effector cell induction are well-understood, potential longer-term effects of these compounds on posttherapy regulatory rebound have not been investigated.

Among the numerous cytotoxic drugs that have been evaluated, cyclophosphamide (CY) has been consistently shown to synergize with immune therapy via its cytotoxic activity on T suppressor cells (15) and, more recently, its proimmunogenic effects on myeloid cells (16, 17). Specifically, these studies demonstrated that a single bolus of CY administered prior to tumor vaccination potentiated both the initial effector T cell priming and the long-term memory response (9, 10, 13, 15). At the same time, whether administration of CY could also diminish the posttherapy T suppressor cell rebound, which rapidly curbs the initial effector activation in-
dependent of its intensity, has not been tested. The potential impact of CY on the posttherapy regulatory surge is particularly relevant in the chronic treatment setting, because repeated immune stimulation leads to exacerbation of counter-regulation and the complete loss of the ability to rescue T effector cell activity (2). To this end, functional kinetics of intratumoral T effector and T suppressor cells, as well as overall tumor growth, were monitored in her-2/neu transgenic FVBNeuN mice following immuno-therapy versus chemoimmunotherapy. The results demonstrated that administration of a single pulse of CY prior to IL-12/GM-CSF treatment resulted in effective depletion of tumor and TDLN-resident T suppressors and enhanced effector T cell activation, as well as delayed the posttherapy rebound and extended the antitumor effector window. Importantly, chronic CY plus IL-12/GM-CSF treatment repeatedly rescued antitumor CTL activity via persistent blockade of the T suppressor upsurge, ultimately resulting in the complete eradication of advanced spontaneous mammary tumors in a significant proportion of the mice.

Materials and Methods

Mice
Breeding pairs of FVBNeuN mice (FVB/N-TgN MMtvN>202Mul) were obtained from Jackson Laboratories (Bar Harbor, ME) and were maintained in our facility. Spontaneous mammary tumor development in female mice was monitored by weekly palpation of animals aged ≥18 wk. Mice were used in studies when the primary tumor reached 100–350 mm³. All studies were approved by the Institutional Animal Care and Use Committee at The State University of New York at Buffalo.

Cytokines and microspheres
Recombinant murine IL-12 was a gift from Wyeth Pharmaceuticals. Recombinant murine GM-CSF was purchased from PeproTech. Phase-inversion nanoencapsulation technology was used to encapsulate IL-12 and GM-CSF in poly-lactic acid microspheres, as previously described, except at a cytokine loading of 0.025% w/v (18). Blank microspheres were used for control injections. Mice were injected intratumorally with 4 mg each microsphere preparation (equivalent to 1 μg each cytokine) suspended in 0.1 ml sterile PBS.

Chemotherapy
CY monohydrate, ISOPAC was purchased from Sigma-Aldrich (St. Louis, MO). Mouse weight was determined, and 200 mg/kg CY (dissolved in sterile PBS) was injected i.p. When combined with microsphere treatment, chemotherapy was administered 24 h prior to IL-12/GM-CSF microspheres.

Monitoring of survival and tumor growth
Tumor size was measured weekly. Two separate diameter measurements of the tumor were recorded using calipers, and the tumor volume was determined by the following formula (A × B²/2 = volume, where A = longest tumor diameter and B = smaller perpendicular tumor diameter. Mice were sacrificed when the longest diameter of the primary tumor reached 15 mm or when the total of the largest diameters of multiple tumors reached 15 mm.

Quantitative real-time PCR
Quantitative real-time PCR was performed as previously described (19). Briefly, total RNA was isolated from tissue samples using TRizol and was reverse transcribed with TaqMan kit (Applied Biosystems). Real-time PCR analysis was performed with the primers listed below and iQ SYBRGreen Supermix (Bio-Rad) using the recommended cycling conditions (denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s). Transcript levels were calculated by the comparative threshold cycle (Ct) method (20). The relative target quantity was normalized to an endogenous control (β-actin) to generate the ΔCt. The ΔΔCt was determined as the ΔCt of samples for the target gene minus the ΔCt of the untreated calibrator for the target gene. Fold change in mRNA level was defined as 2ΔΔCt. The primer sequences were: CDS, 5'-GCC ACA GGA GCC CAT CTA CGA-3' (forward) and 5'-TGG TGT CAT AGA ATG TCT-3' (reverse); granzyme B, 5'-ATC AAG GAT CAG CAG CCT GA-3' (forward) and 5'-TGA TTG CAT CGG TGA AGT TCT-3' (reverse); IFN-γ, 5'-GGG ACA GTC ATT GAA AGC-3' (forward) and 5'-TGG CAG TTT CTC CAG AFA-3' (reverse); and β-actin, 5'-TCA ACC ACA GGC CAT CTA CGA-3' (reverse) and 5'-TGG TGA AGC TGT AGC CAC GCT-3' (reverse).

Flow cytometry and intracellular staining

BrDU injections
BrDU (1 mg), obtained from the BD Pharmingen BrdU flow kit, was injected i.p. 3 h before sacrifice (3).

Flow cytometry and intracellular staining
Mice bearing single established primary tumors (100–300 mm³) were left untreated or received a single cycle of immunotherapy or chemoimmunotherapy. Three weeks later, animals were injected s.c. with the syngeneic LAP0297 lung tumor cell line (kindly provided by Dr. Peigen Huang, Harvard University, Cambridge, MA) and the her-2–expressing NT2.5 mammary tumor cell line (kindly provided by Dr. Elizabeth Jaffee, The Johns Hopkins University, Baltimore, MD) in opposite flanks (2 × 10⁶ cells in 0.1 ml saline, s.c.). Tumor volume was monitored as described above.

Statistical analysis
The Student t test was used to determine the significance of the differences between control and experimental groups in pairwise comparisons. In experiments with multiple groups, homogeneity of intergroup variance was analyzed by ANOVA. Log-rank analysis was used to determine significance in survival studies. In all analyses, p ≤ 0.05 was considered significant.

Results

Administration of CY prior to IL-12 plus GM-CSF treatment delays posttherapy T suppressor cell rebound and enhances both the intensity and the duration of the cytotoxic effector window
The mammary tumors that spontaneously arise in her-2/neu transgenic FVBNeuN mice display a chronic slow-growing phenotype, yet are highly resistant to immune therapy (21–24). Previous studies in our laboratory showed that a single intratumoral injection of slow-release IL-12 and GM-CSF formulations resulted in T cell-dependent regression of established tumors in this model (25). However, the antitumor effect was transient, and tumors resumed growth after several weeks (25). Further analysis
revealed that tumor resurgence was associated with a dramatic expansion of T suppressor cells (2). Fig. 1A displays the cellular kinetics of CD8+ T effector and CD4+ Foxp3+ T suppressor cells in posttherapy tumors, demonstrating the rapid and potent nature of counter-regulation. We hypothesized that elimination of pre-existing CD4+ Foxp3+ T cells prior to treatment could delay treatment-associated regulatory surge. The ability of CY to preferentially deplete regulatory cells is well-known and is thought to be associated with the higher rate of turnover that these cells exhibit in the steady-state tumor. Fig. 1B shows that both TDLN and intratumoral T suppressors proliferated at a significantly higher rate than did CD8+ or CD4+ T effectors. In contrast, administration of CY resulted in a rapid and significant decrease in both the CD4+ Foxp3+/CD8+ T cell and CD4+ Foxp3+/CD4+ Foxp3- T cell ratios on day 3, which remained low until day 8 (Fig. 1C), suggesting that this strategy could be effective in blocking posttherapy T suppressor cell expansion.

To directly test the above hypothesis, mice were administered CY 1 d prior to cytokine treatment, and intratumoral CD8+ T effector versus CD4+ Foxp3+ T suppressor cell kinetics were monitored. The results are shown in Fig. 2. Chemoimmunotherapy had a significant effect on both the effector and the suppressor T cell kinetics (Fig. 2A). CD8+ T cell expansion in mice receiving CY was delayed but ultimately enhanced. Importantly, CD8+ T cells continued to proliferate between days 3 and 7 in the CY plus IL-12/GM-CSF group, whereas proliferation peaked on day 3 but returned to background levels on day 7 in the immunotherapy group. Conversely, chemoimmunotherapy resulted in a 4-fold decrease in CD4+ Foxp3+ T cells on day 3 versus a 6-fold increase in T suppressors in mice receiving immunotherapy alone (Fig. 2C). However, CY-mediated reduction in T suppressor cell numbers on day 3 was short-lived, and the cells recovered rapidly between days 3 and 7, reaching levels that were observed in the immune therapy-alone group.

The ratio of proliferating CD8+ T cells/CD4+ Foxp3+ T cells in the tumor (i.e., the activity index) is highly predictive of therapeutic efficacy (3). To this end, monitoring of the activity index in mice receiving chemoimmunotherapy revealed that effector activity increased nearly 5-fold between days 0 and 3 but declined rapidly thereafter, reflecting the rapid recovery of T suppressor cells between days 3 and 7 (Fig. 2B). In contrast, in the immune therapy-alone group, activity index remained unchanged between days

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Preferential elimination of CD4+ Foxp3+ T suppressor cells from tumors and TDLN of FVBNeuN mice. A. Intratumoral IL-12/GM-CSF induces T suppressor cell expansion. Mice were sacrificed prior to (day 0) or 1, 3, 7, and 14 d after intratumoral IL-12/GM-CSF microsphere treatment, and tumor/TDLN T cell populations were quantified. Both CD8+ T and CD4+ Foxp3+ T cells increased in numbers following treatment compared with pretherapy levels. Asterisks indicate significance compared to day 0 (p = 0.01 and p = 0.002 for CD8+ T cells on day 3 and for CD4+ Foxp3+ T cells on day 7, respectively; n = 6–9 mice/group). B, T suppressor cells proliferate at a higher rate than do T effector cells. In vivo proliferation was assessed by BrdU uptake. CD4+ Foxp3+ cells proliferated at a significantly higher rate than did CD4+ Foxp3- or CD8+ T cells both in the tumor and the TDLN. Asterisks indicate significance compared to effector cells (p ≤ 0.02; n = 9 mice/group). C, CY mediates preferential and long-lasting T suppressor cell depletion. Mice were sacrificed prior to (day 0) or 1, 3, 5, and 8 d following CY administration. The ratios of CD4+ Foxp3+/CD8+ T cells and CD4+ Foxp3+/CD4+ Foxp3- T cells decreased in both the TDLN (p ≤ 0.01) and the tumor (p ≤ 0.03; n = 6–11 mice/group). Asterisks indicate statistical significance compared to day 0. Errors bars represent SE.
and 3 and declined to below pretherapy levels on day 7, demonstrating the potent neutralizing effect of the T suppressor cell expansion (Fig. 2B). The advantage that chemoimmunotherapy provides is best displayed in Fig. 2C, in which the fold differences between the activity indices of the two groups are displayed. These data demonstrated that CY enhanced the activity index by 4-fold on day 3 and by 7-fold on day 7, with the ratio returning to background levels on day 14. The above findings established that chemotherapy delayed the T suppressor cell rebound and enhanced the intensity and the duration of the effector window.

Chronic chemoimmunotherapy achieves complete cure of advanced spontaneous mammary tumors

Earlier studies in FVBnNeuN mice showed that repeated treatment could revive effector activity but that the intensification of T suppressor cell resurgence with each cycle of treatment ultimately led to loss of therapeutic benefit (2). To this end, we next tested the hypothesis that chronic chemoimmunotherapy could maintain the activity of antitumor CD8\(^+\) T effectors and achieve superior tumor eradication in the long-term. Mice with established spontaneous mammary tumors were treated with control particles, CY alone,
IL-12/GM-CSF particles, or CY plus IL-12/GM-CSF particles every 3 wk for three cycles, and tumor growth was monitored. Tumor-growth patterns of individual mice in different groups are shown in Fig. 3A. These data revealed that tumors grew progressively in the control mice, and all but one mouse had to be euthanized by week 9. Treatment with CY alone caused a slight delay, but all tumors maintained growth. IL-12/GM-CSF therapy achieved longer-term suppression, with tumors regressing completely in 2 of 11 mice during therapy; however, both tumors recurred by week 9. In contrast, seven of nine tumors either regressed or had their growth arrested for the duration of the study in the chemoimmunotherapy group, with complete long-term cure in four of seven responders. The overall survival patterns are shown in Fig. 3B. In the chemoimmunotherapy group, all mice remained alive at week nine. In contrast, 9-wk survival was 72, 55, and 10% in immune therapy, CY-alone, and control groups, respectively. All remaining mice in these groups were euthanized between weeks 9 and 10 due to continued primary tumor growth or the development of independent secondary tumors. Of the four “cured” mice in the chemoimmunotherapy group, one mouse at week 12, whereas the other three remained tumor-free for 3 mo, at which point they were euthanized. Separately, immune therapy alone failed to suppress the development of secondary tumors, but CY alone or
CY plus IL-12/GM-CSF had a substantial impact on the appearance of independent tumors (Fig. 3C). Collectively, these results established that chemoimmunotherapy was highly effective in the chronic treatment setting.

Curative effect of chronic therapy is associated with maintenance of CD8+ T cell cytotoxicity

Although the data shown in Fig. 3 confirmed the long-term efficacy of the combination strategy, it was not clear whether this effect was due to enhanced CD8+ T cell activity or was associated with T cell-independent effects of CY. To this end, T cell cytotoxicity markers were monitored in posttherapy tumors. The expression levels of CD8, IFN-γ, and granzyme B mRNA were monitored on day 3 posttreatment in the control, immune therapy, and chemo-immunotherapy groups. Immune therapy alone induced increases in both IFN-γ (30-fold) and granzyme B (2-fold) levels when normalized to CD8 expression (Fig. 4A). In contrast, chemo-immunotherapy resulted in significantly superior cytotoxic activity, with 80- and 6-fold increases in IFN-γ and granzyme B, respectively (Fig. 4A). These increases were primarily associated with CD8+ T cell activation, because depletion of CD8+ T cells prior to treatment resulted in the near-complete loss of posttherapy cytotoxicity (Fig. 4B).

The results above established an empirical link between preferential T suppressor cell depletion and enhanced CD8+ T cell activity. However, CY can promote CTL cytotoxicity via other mechanisms, including the induction of a homeostatic recovery environment (14), the abrogation of tumor-associated macrophage- or myeloid-derived suppressor cell-suppressive function (16, 17), and/or the promotion of myeloid cell inflammatory activity (26, 27). To this end, we tested a more targeted depletion strategy involving the use of anti-CD25 Ab, which selectively eliminates pre-existing CD4+ CD25+ T suppressor cells when administered prior to T effector activation (28). Analysis of the activity index, as well as tumor growth in mice treated with the anti-CD25 Ab versus CY, demonstrated that both strategies were equally effective in enhancing tumor suppression, confirming the direct link between T suppressor cell elimination and enhanced antitumor cytotoxicity (Supplemental Fig. 1). Separately, we also found that the nonspecific proinflammatory activity of CY (26, 27) was not a contributing factor, because addition of CY to IL-12/ GM-CSF treatment did not enhance IL-1β production, a marker of

![FIGURE 4. Effect of chemoimmunotherapy on the function and antitumor role of CD8+ T cells. A, CY augments posttherapy cytotoxicity. IFN-γ and granzyme B mRNAs were quantified by real-time PCR prior to and 3 d postmicrosphere treatment and normalized to CD8 mRNA. The differences between the chemoimmunotherapy group and immune therapy-alone or control groups were significant (asterisks) both for IFN-γ and granzyme B (p < 0.02; n = 8–9 mice/group). B, CY-mediated enhancement of cytotoxicity is dependent on CD8+ T cells. The effect of CD8+ T cell depletion on IFN-γ and granzyme B expression was determined. The pairwise differences between the CD8+ T cell-depleted experimental and nondepleted control groups were significant (*p < 0.02; n = 3–6 mice/group). C, Chemoimmunotherapy restores CD8+ T cell-mediated tumor kill. Mice were depleted of CD8+ T or NK cells prior to treatment, as described in Materials and Methods. Fold change in tumor volume was determined 3 wk postmicrosphere injection. Fold change in tumor volume in all treatment groups was significant compared with control (p < 0.002; n = 6–14 mice/group in the CD8-depletion study; n = 5–14 mice/group in the NK cell-depletion study). Error bars represent SE. The overall pattern of tumor growth remained essentially identical when actual tumor volumes were plotted (Supplemental Fig. 2).](http://www.jimmunol.org/)
Previous studies in FVBneuN mice demonstrated only a partial role for CD8\(^+\) T cells in IL-12-GM-CSF-mediated tumor regression (25). In addition, a rapid, but transient, activation of NK cells was also found to be critical to post–IL-12-GM-CSF tumor kill (19). Because the above data revealed a significant enhancement of CD8\(^+\) T cell cytotoxicity by CY, in vivo Ab depletions were performed to determine the respective roles of CD8\(^+\) T and NK cell subsets in CY plus IL-12/GM-CSF-mediated tumor cure. The results are shown in Fig. 4C. Immune therapy alone resulted in moderate suppression of tumor growth. Depletion of CD8\(^+\) T cells did not significantly impact tumor suppression, suggesting that CD8\(^+\) T cells did not play a major role in tumor kill following treatment with IL-12 plus GM-CSF alone. In contrast, depletion of the NK cells resulted in a significant loss (~50%) of tumor suppression. As expected, chemoimmunotherapy resulted in a dramatic enhancement of tumor suppression. However, in contrast to what was observed with immune therapy alone, depletion of CD8\(^+\) T cells completely nullified the CY-mediated increase in antitumor activity. The augmentation of CTL cytotoxicity by CY was not due to direct stimulation of CD8\(^+\) T cells, because depletion of CD8\(^+\) T cells in mice receiving CY alone did not adversely affect the transient antitumor activity of CY (data not shown). Moreover, depletion of NK cells in these mice still resulted in potent tumor suppression, identifying CD8\(^+\) T cells as the primary antitumor effectors in this setting. These data established that the synergistic effect of CY was strictly associated with the restoration of CD8\(^+\) T cell-mediated tumor kill.

Although these findings established the critical role of CD8\(^+\) T cell activation in chemoimmunotherapy-mediated tumor kill, it was not determined whether this activity resulted in the development of long-term protective memory. To address this question, FVBneuN mice bearing single established spontaneous tumors were treated either with IL-12-GM-CSF or CY plus IL-12/ GM-CSF and were then challenged with a her-2–expressing FVBneuN mammary tumor cell line (NT2.5) and a syngeneic control lung tumor cell line (LAP0297). The mice were then monitored for tumor growth. The data shown in Table I established that treatment of the primary tumor prior to challenge resulted in the development of a tumor-specific response that effectively suppressed the growth of the syngeneic FVBneuN mammary tumor cell line but not that of the lung tumor cell line (the somewhat slower growth of the lung tumor in the treated mice was not significantly different from that observed in the control mice). Furthermore, the protection afforded by chemoimmunotherapy was highly superior to that induced by immune therapy alone.

To determine whether CD8\(^+\) T cell activity was maintained during repeated treatment, quantitative and qualitative changes in CD8\(^+\) T cell and CD4\(^+\) Foxp3\(^+\) T suppressor cells were monitored in mice receiving multiple treatments. Tumors were analyzed 3 d after the third treatment because previous analyses had revealed that the activity index peaked on day 3 posttherapy (Fig. 2). Both CD8\(^+\) T cell and CD4\(^+\) Foxp3\(^+\) T cell numbers increased by an average of 4–6-fold in mice treated with immune therapy alone, similar to that seen following the first treatment (Fig. 5A). In contrast, CD8\(^+\) T cell numbers remained unchanged, whereas the CD4\(^+\) Foxp3\(^+\) T cell numbers decreased by an average of 3-fold after the third treatment, again replicating the pattern observed after the first treatment. Moreover, chemoimmunotherapy again enhanced both the CD8\(^+\) T cell proliferation and the activity index (Fig. 5B). Finally, chemoimmunotherapy induced a 2–3-fold increase in CD8\(^+\) T cell cytotoxicity markers in comparison with immune therapy alone (Fig. 5C). These data established that chronic therapy repeatedly rescued T effector cell activity during the 6-wk therapeutic window, although the overall enhancement diminished after the third cycle.

### Discussion

Our data demonstrated that administration of a single pulse of CY prior to IL-12-GM-CSF therapy promoted complete cure of advanced spontaneous mammary tumors in FVBneuN mice, a model in which therapeutic success has been limited to either the preventive setting (21–24) or to treatment of transplanted syngeneic tumor cell lines (29–32). The potentiation of tumor kill was directly associated with depletion of the pre-existing T suppressor cell pool, resulting in a transient delay in posttherapy T suppressor cell expansion and an extended cytotoxic window. Importantly, this approach allowed repeated rescue of CD8\(^+\) T cell cytotoxicity leading to long-term benefit. This is in contrast to earlier studies in which the intensification of the counter-regulatory response during chronic treatment with IL-12-GM-CSF particles culminated in the complete loss of antitumor CTL activity (2).

Previous work had established that IL-12-GM-CSF treatment could induce tumor-specific CTL in FVBneuN mice, which could effectively kill autochthonous mammary tumors in culture (2, 25). At the same time, Ab-mediated subset-depletion studies suggested a modest role for CD8\(^+\) T cells in treatment-induced tumor regression in vivo (25). These findings led to the hypothesis that failure of posttreatment CTL to mediate successful tumor regression in vivo was likely associated with the concurrent expansion of CD4\(^+\) CD25\(^+\) Foxp3\(^+\) T suppressor cells (2). The current findings support this notion because transient neutralization of the regulatory rebound resulted in the restoration of CD8\(^+\) T cell-mediated tumor kill, demonstrating the critical role of these cells in long-term tumor eradication. The finding that NK cells could mediate effective tumor suppression, independent of posttherapy T suppressor cell expansion, in earlier studies was likely associated with the rapid kinetics of post-IL-12 NK cell activation in the tumor (19), which precedes posttreatment CD8\(^+\) T cell priming (33) and T suppressor cell expansion (3).

Analysis of latent tumor development in posttherapy mice revealed that primary and secondary tumors responded differently to treatment. Specifically, although immunotherapy was critical to suppression of primary tumors, secondary tumors were suppressed in CY-alone or CY plus IL-12-GM-CSF groups but not in the IL-12/ GM-CSF–alone group. Systemic suppression of treatment-induced

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**Table 1. Development of a tumor-specific protective memory response in posttherapy mice**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Control</th>
<th>IL-12/GM-CSF</th>
<th>CY + IL-12/GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAP0297</td>
<td>430 ± 169</td>
<td>238 ± 79</td>
<td>268 ± 58</td>
</tr>
<tr>
<td>NT2.5</td>
<td>233 ± 58</td>
<td>146 ± 41</td>
<td>34 ± 40</td>
</tr>
</tbody>
</table>

* Tumors were measured 2 wk postchallenge, at which time the mice were euthanized because of primary tumor outgrowth. Average tumor volume ± SD is shown (n = 5 mice/group).
  
* The differences between control and treatment groups were not significant for the LAP0297 cell line (p ≥ 0.15).
  
* The differences between control and IL-12/GM-CSF groups were significant (p = 0.038).
  
* The differences between control versus CY + IL-12/GM-CSF and IL-12/GM-CSF versus CY + IL-12/GM-CSF groups were significant (p = 0.002 and p = 0.005, respectively). Tumors were rejected in three of five mice in the CY + IL-12/GM-CSF group.
CTL by expanding T suppressors in mice receiving immune therapy alone could explain this observation. Alternatively, because multifocal tumors arise independently in FVBNeuN mice, the CTL that are induced against the treated tumor may not be effective against secondary tumors with different immunogenic profiles (34). Separately, suppression of secondary tumors by CY could be due to its immune-independent cytotoxic effects or to its ability to enhance concomitant immunity (35), particularly because the latent tumor burden was minimal (i.e., nonpalpable) at the time of the initial treatment. Whether the observed differences

FIGURE 5. Effect of chronic chemoimmunotherapy on CD8+ T cell effector function. Mice were treated every 3 wk for a total of three treatments (microsphere injections on days 0, 21, and 42). Tumors were analyzed 3 d after the third treatment (day 45). Fold changes for each parameter shown were determined via comparison of day 45 tumors with control tumors obtained from mice that received two treatments but were euthanized just prior to the third microsphere treatment. Each symbol represents an individual mouse. A, Repeated treatment maintains preferential T suppressor cell depletion. The differences between the immune therapy and chemoimmunotherapy groups were significant both for CD8+ and CD4+ Foxp3+ T cells. B, CD8+ T cell activation is preserved during chronic therapy. CD8+ T cell proliferation and the activity index were analyzed after the third treatment. The differences between immune therapy and chemoimmunotherapy groups were significant for proliferation and activity index. Chauvenet criterion was applied to account for a single outlier in this study. Horizontal bars represent averages (n = 8–9 mice/group). C, Repeated treatment maintains CD8+ T cell cytotoxicity. IFN-γ and granzyme B mRNA levels were determined and normalized to CD8 mRNA in control (day 42) and treatment (day 45) groups. Post-treatment IFN-γ and granzyme B levels in the chemoimmunotherapy group were significantly different from control (p ≤ 0.01). No significance was obtained for the immune therapy group. Error bars represent SE (n = 7–9 mice/group).
are due to one or a combination of the above remains to be determined.

A direct comparison of the intensities of posttherapy CD8+ T cell activity after the first and third treatments revealed that, although the elimination of pre-existing T suppressors allowed repeated rescue of T effector activity, overall cytotoxicity diminished with additional treatment. It is well-known that chronic exposure to Ag can result in the anergy or deletion of effector T cells (36, 37). Moreover, the preferential effects of CY on T suppressor cells are not absolute, and collateral CD8+ T cell loss cannot be avoided (28). It was not determined whether the progressive loss of CTL cytotoxicity is associated with T cell-intrinsic exhaustion/deletion mechanisms, CY-mediated cumulative loss of antitumor CD8+ T cells, or a combination of both. Preliminary quantification of CTLA-4, programmed death 1, or carcioembryonic Ag-related cell adhesion molecule 1 on tumor-infiltrating CD8+ T cells demonstrated a 2–3-fold increase in membrane expression after the first treatment cycle; however, additional treatments failed to induce further change (J.L. Harden and N.K. Egyházi, unpublished observations), suggesting that T cell-intrinsic negative-checkpoint mechanisms did not play a major role in the long-term loss of T cell cytotoxicity. Regardless of the underlying mechanism, our findings suggested that, even when posttherapy regulation is blocked, progressive contraction of the T effector cell pool during chronic treatment may ultimately limit the long-term usefulness of immunotherapy in the management of tumors that persist.

Disclosures
N.K.E. has ownership interest in TherapyX, Inc., which is developing sustained-release formulations of cytokines for cancer therapy.

References


Supplemental Figure 1. Comparison of anti-CD25 antibody and CY. Panel A. Mice were either treated with anti-CD25 antibody or CY prior to IL-12/GM-CSF treatment (methods). The activity indices (proliferating CD8+ T-cell number / CD4+ CD25+ Foxp3+ T-suppressor cell number) for the TDLN and tumors are shown. Analysis was performed 3 days post-treatment. Control = blank microspheres, IT = immunotherapy (IL-12/GM-CSF), CY = cyclophosphamide. Error bars = SE, n = 5-9 per group. Anti-CD25 + IT group was significantly different from control and IT groups in both the tumor and the TDLN (p< 0.016). Panel B. Fold-change in tumor volume between days 0 (treatment) and 21 are shown. Both CY + IT and anti-CD25 + IT groups were significantly different from control as well as IT groups (p < 0.038). Error bars = SE, n = 5 – 14 per group.
Supplemental Figure 2. Chemoimmunotherapy restores CD8+ T-cell-mediated tumor kill. The data displayed in Figure 4C were re-plotted using actual tumor volumes. Tumor-bearing mice (starting tumor volume of 100-350 mm³) were depleted of CD8+ T or NK-cells prior to treatment as described in the Methods. Tumor volume was determined 3 weeks post-microsphere injection. Differences in tumor volume in all treatment groups was significant compared to control (p < 0.007) except for the IL-12/GM-CSF treatment with CD8+ T-cell or NK-cell depletions (p > 0.08). Error bars = SE, n = 6-14 per group in the CD8 depletion study and n = 5-14 per group in the NK-cell depletion study.