

Partial, but not Complete, Tumor-Debulking Surgery Promotes Protective Antitumor Memory when Combined with Chemotherapy and Adjuvant Immunotherapy

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Abstract

Resection alone is rarely curative for advanced tumors, but the outcome generally improves with adjuvant therapy. We have previously shown that a combination of traditional chemotherapy (gemcitabine) and immunotherapy (anti-CD40/FGK-45) without surgery is synergistic and can lead to long-term cure when applied to small tumors. Such cured animals have immunologic memory and are protected from rechallenge. Here we investigate the effectiveness of combination chemotherapy and immunotherapy after partial or complete surgical debulking of large tumors. We found that complete resection followed by combination chemotherapy/immunotherapy led to a high rate of cure (>80%) but failed to induce a long-term, tumor-specific memory. Partial debulking followed by combination therapy elicited the same proportion of cured animals but in contrast to complete resection, a memory response was invoked. We postulate that chemotherapy induced apoptosis of the residual tumor cells following incomplete resection is absolutely required for the induction of long-term immunologic memory. (Cancer Res 2005; 65(17): 7580-4)

Introduction

Surgery for solid cancers often fails to cure the patient because of local recurrence or because micrometastases that were undetectable at the time of surgery, subsequently grow. Thus, postoperative adjuvant therapies such as immunotherapy and chemotherapy have been used in the hope that they will destroy any residual tumor cells. Such strategies have met with limited success. Therefore, patients with extensive disease have been typically made ineligible for surgery on the basis that the procedure would not be able to remove some part of the tumor. We wanted to know if there was a sound scientific basis for these assumptions and set out to test the relative efficacy of adjuvant therapies in the context of complete or partial resections. Our results show that incomplete tumor resection provides a better environment to generate protective antitumor memory responses than complete resection. Partial tumor resection followed by *both* chemotherapy and immunotherapy yielded a high cure rate and long-lived antitumor memory. In contrast, when the tumor was completely resected, we found that postoperative combination therapy did not

lead to antitumor memory. Thus, the persistence of antigen after incomplete debulking (in the form of apoptotic tumor cells) in combination with immunotherapy is critical for the induction of long-term immunity. These observations have implications for the way surgery, immunotherapy, and chemotherapy are used in cancer treatment protocols.

Materials and Methods

Mice. BALB/c (H-2^d) mice were obtained from the Animal Resources Centre (Perth, Western Australia) and maintained under standard conditions. All mice used in these experiments were between 6 and 8 weeks of age. Animal experiments were carried out according to protocols approved by the University of Western Australia Animal Ethics Committee.

Cell lines. The murine malignant mesothelioma cell line (AB1) was generated by injecting crocidolite asbestos i.p. into BALB/c mice (1). It was subsequently transfected with the influenza virus hemagglutinin (*HA*) gene (2). Expression of HA on the cell line AB1-HA was verified by fluorescence-activated cell sorting (FACS) analysis before use in experiments.

Experimental protocol. Viable AB1-HA cells (1×10^6) were inoculated s.c. into the ventral surface in the lower flank region. Surgery was done on day 20 after inoculation, when the average tumor size was around 50 mm². Chemotherapy (gemcitabine) commenced 2 days after surgery. Mice were injected i.p. with 120 μg/g gemcitabine every third day for five doses (q3dx5), a dosing regimen previously established as effective in this model (3). Control mice received the PBS vehicle alone. Immunotherapy was given as anti-CD40 antibody (FGK-45; 100 μg in 200 μL of PBS) i.p. every second day for three doses (q2dx3). CD40 is not expressed on the AB1-HA tumor cells as determined by FACS analysis. The FGK45 antibody had no effect on the rate of growth of tumor cells *in vitro*. Initial experiments showed that normal rat IgG given under a similar dosage regime had no significant effect on tumor growth rates at any time point or on *in vivo* CTL activity in peripheral lymph nodes or spleen when compared with PBS (4); therefore, 200 μL of PBS i.p. were chosen as a control for all subsequent experiments. Mice receiving the combination started therapy 2 days after surgery with 1 day between chemotherapy and immunotherapy. Control mice received PBS or FGK-45 from 2 days postsurgery for three doses. Tumor size was monitored with calipers thrice weekly until tumor size reached 10 × 10 mm, when mice were euthanized. Surviving mice were rechallenged with viable AB1-HA tumor cells (1×10^6) contralaterally as above at day 90 postsurgery. Tumor size was measured as described.

Surgical resection of tumors. Mice were anaesthetized with 3.6% chloral hydrate 0.1 mL/10 g mouse i.p. and inhaled methoxyflurane as necessary. Debulking surgery was done via elliptic incisions, centered over the s.c. tumors. Skin flaps were then elevated to expose adherent tumors. Once tumors were dissected clear of adjacent fascia, debulking was done, with preservation of the tumor pedicles. Wounds were closed primarily, using staples (LT-100 liga clips, Ethicon, North Ryde, Australia), or 5/0 vicryl (polyglactin 910, Ethicon) interrupted sutures. Mice received 2.5 mg/kg buprenorphine i.p. in the recovery phase for postoperative analgesia as required.

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Statistical analysis. Survival curves were compared by the log-rank test. Groups were compared using Fisher's exact test. Differences were considered significant when $P < 0.05$. Statistical analysis was conducted using GraphPad Prism and GraphPad InStat.

Results and Discussion

Small tumor deposits such as those that remain after incomplete surgery may be vulnerable to immune attack but only if tumor-specific effector T cells can be rapidly induced. The *de novo* induction of these T cells from naive precursors is thought to be too weak and/or slow to achieve this (5). This implies that immunotherapy can only be successful in the postsurgical environment if the primary tumor generates an appropriate memory T-cell response. As a single protocol, immunotherapy has met with only limited success but may be boosted by combination with other approaches including chemotherapy.

Combination of chemotherapy and immunotherapy induces regression and cure of a partially resected tumor. We set out to determine whether the notoriously poor prognosis after partial debulking surgery could be improved with subsequent chemotherapy and/or immunotherapy in our mouse model of malignant mesothelioma. In particular, we focused on determining how treatment affected the generation of antitumor immunologic memory. Mice were inoculated with AB1-HA mesothelioma cells and were resected 20 days later (Fig. 1A) when the mean tumor size was 50 mm². Most of the tumor was removed to leave ~25% *in situ*. The intention was to model the level of removal that might be expected to occur in a patient with mesothelioma who might be currently determined to be ineligible for such a surgical procedure. This degree of debulking in the absence of any other therapy (vehicle) was not curative as might be predicted. None of the 12 mice in this group survived past day 40 (Fig. 1B). Mice given antigen-presenting cell (APC)-directed immunotherapy (CD40 ligation with the monoclonal antibody FGK-45) after surgery showed a small growth delay (<20 days; Fig. 1B) compared with vehicle-treated mice ($P < 0.05$). However, this treatment did not improve survival. None of the 10 mice in this group remained tumor free. Chemotherapy (gemcitabine) after surgery produced slightly better results: mice treated with gemcitabine alone survived significantly longer ($P < 0.05$) than both FGK-45 alone and vehicle-treated mice, with 2 of 10 mice surviving long-term tumor free (Fig. 1B). One of these two survivors resisted tumor rechallenge, which is indicative of persisting antitumor memory (Fig. 1C).

When partially resected mice were treated sequentially with chemotherapy then immunotherapy, six of nine mice showed long-term tumor-free survival. The three animals that were not cured by this combination therapy all showed a delay in primary tumor growth (data not shown). Importantly, five of the six long-term survivors of triple therapy were protected from subsequent rechallenge (Fig. 1B and C).

These data extend our previous observations in which we found that gemcitabine-mediated chemotherapy was highly synergistic with an APC-directed CD40 signal in the treatment of small tumors, leading to an unprecedented (80%) cure rate (6). We have also shown that the immune priming ability of chemotherapy can be explained, at least in part, by the increase in the amount of tumor antigens arriving in the draining lymph node (7). Apoptotic tumor cells provide increased levels of cross-presented tumor antigen in the draining lymph nodes and this can be exploited

therapeutically using an adjuvant immunotherapy designed to activate the APCs.

Combination therapy does not induce immunologic memory when tumor is completely resected. To establish whether the presence of tumor and therefore tumor antigens in the post-surgery therapeutic environment was an important factor in promoting antitumor memory, we now did experiments in which the entire tumor was removed followed by combination therapy. Complete resection in the context of this investigation required that all macroscopically identifiable tumors be excised. Completely resected mice treated with combination therapy showed a significant increase ($P < 0.05$) in long-term tumor-free survival over completely resected mice subsequently treated with vehicle alone, with 13 of 16 mice (81%) free of tumor to day 110 after tumor inoculation (Fig. 2A). However, none of these 13 survivors was able to resist rechallenge suggesting that no immunologic memory had developed.

Complete resection (vehicle) was a reasonably effective single procedure with 4 of 16 (25%) mice surviving long-term tumor free (Fig. 2A). The 12 mice that regrew tumors after surgery without further therapy did display some retardation of tumor growth in

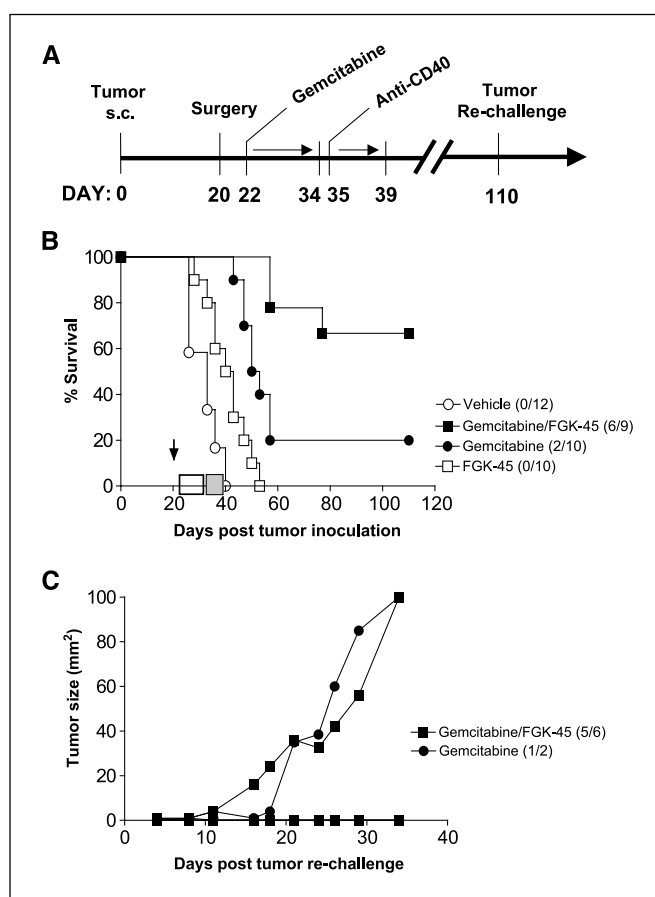


Figure 1. Partial surgical debulking combined with chemotherapy/immunotherapy promotes memory/survival after rechallenge. **A**, BALB/c mice bearing AB1-HA tumors were partially (75%) surgically debulked of tumor then treated with chemotherapy and immunotherapy as indicated in the timeline. Surviving mice were rechallenged (1×10^6 viable tumor cells) contralaterally at day 110. **B**, survival curves after partial resection and further treatment as indicated; vehicle, no further treatment; gemcitabine/FGK-45, chemotherapy and immunotherapy; gemcitabine, chemotherapy alone; FGK-45, immunotherapy alone. The number of survivors from the total number of animals in each group is indicated in parentheses. **C**, tumor growth curves after rechallenge for individual mice. All survivors remained tumor free.

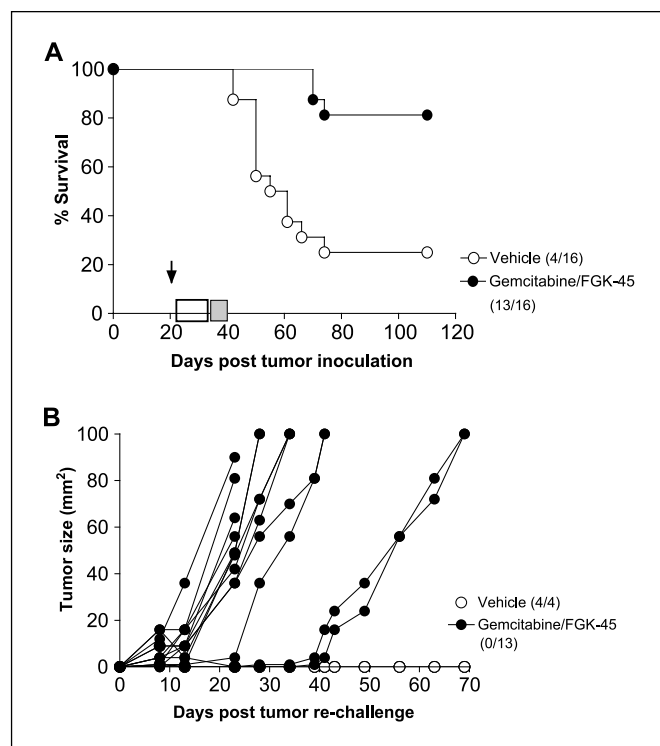


Figure 2. Tumor antigen in the form of a partially resected tumor is required after surgery for the combination therapy to effectively enhance long-term survival and promote immunologic memory. BALB/c mice bearing AB1-HA tumors were completely resected and either not treated (vehicle) or treated with combination chemotherapy and immunotherapy; gemcitabine/FGK-45 as indicated. Surviving mice were rechallenged contralaterally at day 110. *A*, survival curves showing the number of survivors from the total number of animals in each group in parentheses. *B*, tumor growth curves after rechallenge for individual mice. All surviving mice remained tumor free.

comparison with normal tumor growth after inoculation of cells. Perhaps surprisingly, all four survivors from single protocol surgery were protected from rechallenge (Fig. 2*B*). This is a reproducible finding. In two other independent experiments where four and five animals survived the complete resection with no additional

therapy, we found that six of these nine animals displayed memory in that they were resistant to a contralateral tumorigenic rechallenge. Part of the explanation for this may be attributed to a release from immunosuppression (8). Unfortunately, there is a distinct paucity of literature about this phenomenon, but it seems clear that removal of a large primary tumor, at least in mice, can reverse immunosuppression (9). Surgery may also unmask a population of primed T cells that can mediate protective immunity (10) and may have the capacity to restore immunologic function even when disseminated metastatic disease is present (11). The finding that memory is differentially invoked in animals that receive adjuvant therapy compared with no therapy after complete resection is highly significantly different yielding $P = 0.0004$ using Fisher's exact test (Table 1).

Immunotherapy does not alter the rate of cure after complete resection with adjuvant chemotherapy. We hypothesized that the enhanced efficacy of the triple therapy protocol in the context of complete resection was in part due to the recognized synergy between chemotherapy and immunotherapy (6). To test this, completely resected mice were then treated with adjuvant chemotherapy with no subsequent immunotherapy (Fig. 3*A*). Sixteen of 18 (89%) of mice treated in this way remained tumor free to day 110 after tumor inoculation (Fig. 3*B*). There was no significant difference between this survival rate and that of the completely resected mice that received combination therapy (Fig. 2*A*). However, there was a significant difference between the tumor growth rates in those mice that were not cured from these two groups, with the two resection/chemotherapy-treated mice succumbing to tumor 15 days before the three triple protocol-treated mice. If we consider the case of complete resection and adjuvant therapy, we detail two experiments. In one, chemotherapy only is used (Fig. 3), and in the other both chemotherapy and immunotherapy are used (Fig. 2). These experiments have identical outcomes, both in terms of the proportion of initial survivors (81% and 89%) and the lack of induction of memory (13% and 0%). These data are not (statistically) significantly different and can be considered as independent repeats of an experiment to test the null hypothesis that adjuvant therapy does not differently affect the induction of memory after complete or partial resection. In both of

Table 1. Summary of survival after primary therapy and resistance to rechallenge

Surgery	Chemotherapy	Immunotherapy	Survival (%)	Memory (%)
Partial	—	—	0/12 (0)	NA
Partial	+	—	2/10 (20)	1/2 (50)
Partial	—	+	0/10 (0)	NA
Partial	+	+	6/9 (67)	5/6 (83)*
Complete	—	—	4/16 (25)	4/4 (100) [†]
Complete	+	—	16/18 (89)	2/16 (13) [‡]
Complete	+	+	13/16 (81)	0/13 (0)

NOTE: Surgery was defined as complete where no visible tumor remained at the primary site. Partial denotes ~75% removal of the primary mass. Chemotherapy consisted of 120 $\mu\text{g/g}$ gemcitabine every third day for five doses (q3dx5).

Immunotherapy was given as anti-CD40 antibody (FGK-45; 100 μg in 200 μL) every second day for three doses (q2dx3). Memory is defined as survival after a tumorigenic rechallenge.

Abbreviation: NA, not applicable.

* $P = 0.0005$, compared with complete resection and combination adjuvant therapy (Fisher's exact test).

[†] $P = 0.0004$, compared with complete resection and combination adjuvant therapy (Fisher's exact test).

[‡] $P = 0.0043$, compared with partial resection and combination adjuvant therapy (Fisher's exact test).

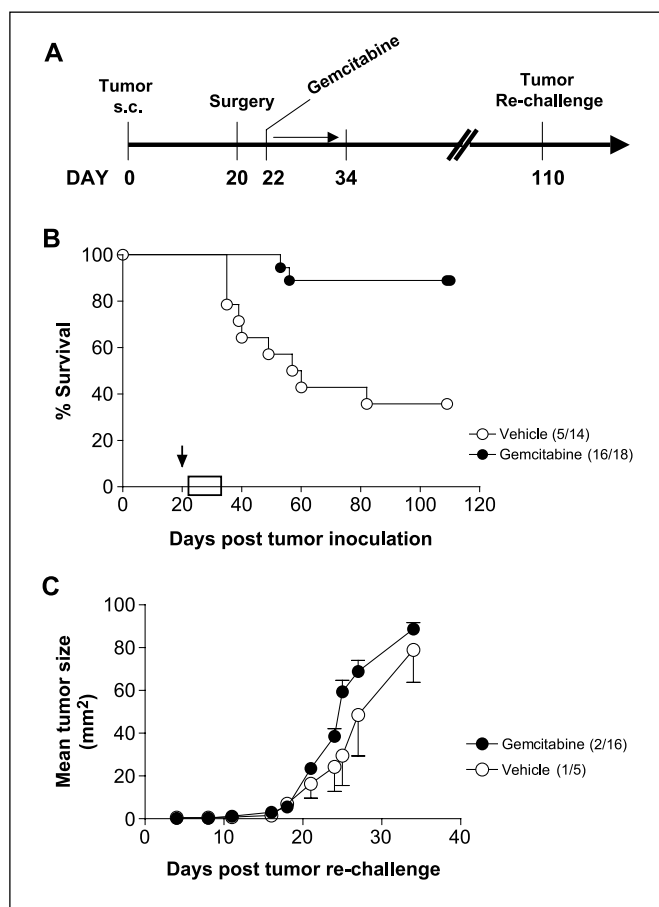


Figure 3. Survival after complete resection is not altered by immunotherapy. **A**, BALB/c mice bearing AB1-HA tumors were completely resected and treated as indicated in the timeline. **B**, survival curves after complete resection only (vehicle) and after complete resection and chemotherapy without subsequent immunotherapy (gemcitabine). The number of survivors from the total number of animals in each group is indicated in parentheses. **C**, tumor growth curves after contralateral rechallenge for those animals where tumors grew. The number of survivors from the total number of animals in each group is indicated in parentheses.

these experiments, the hypothesis is denied (P values enumerated in Table 1).

Finally, we assessed antitumor immunity in the 16 mice that survived the combination of complete resection and chemotherapy, by challenging these mice contralaterally at day 110 after the initial tumor inoculation (Fig. 3C). Of these 16 mice, two mice survived long-term tumor free. The other 14 mice rapidly developed tumor suggesting that this protocol had failed to induce memory. From the vehicle-treated group (those mice that underwent surgery without further therapy), there were five long-term survivors. Only one of these animals was able to resist rechallenge.

Mesothelioma, like many invasive solid tumors is difficult to treat and the surgical reality is that most patients are deemed inappropriate for resection (12). This view holds, at least in part because it is known that in many cases, significant portions of the tumor cannot be excised. In the present study, we show that (i) a reasonable outcome is possible if both chemotherapy and immunotherapy are used after surgery and (ii) that the remaining tumor in this scenario, rather than being detrimental to the outcome can have a positive effect, invoking protective immunity against recurrence. These data suggest that the apparent

disadvantage of incomplete tumor removal can be turned into a benefit when the appropriate combination of chemotherapy and immunotherapy is used after surgery.

We found a synergy between chemotherapy (gemcitabine) and immunotherapy (anti-CD40 treatment) that is consistent with our previous work in which this combination therapy was used to treat small tumors (6). In the present study, we show the relevance of this approach to the treatment of larger, vascularized tumors in combination with surgical debulking. As summarized in Table 1, applying both chemotherapy and immunotherapy after surgery has the capacity to generate antitumor immunity but *only* when a reservoir of tumor antigen is available after the resection (compare groups 4 and 7, Table 1). If, in contrast, the entire tumor mass is removed, we found that post-surgery combination therapy is in fact detrimental. The ability to generate protective long-term memory after complete resection only (group 5) is lost when combination chemoimmunotherapy is subsequently given. This perhaps counterintuitive result probably arises because of the profound lymphopenia that occurs after chemotherapy and suggests that the formation of memory after surgery and combination therapy is absolutely dependent on the presence of antigen at the time of combination treatment. Thus, if a tumor can indeed be completely removed in a clinical setting, combination chemoimmunotherapy should be accompanied by vaccination with autologous tumor antigens, to provide a *de novo* reservoir of tumor antigens. Our results help to provide a set of guidelines that could be applied in the clinic and suggest that some patients with extensive disease, previously deemed unsuitable for surgery might benefit from a partial resection with appropriate adjuvant therapies.

The concept that incomplete resection could generate a better antitumor memory response than complete resection is consistent with the idea of "concomitant immunity." Concomitant immunity is an old idea that describes the phenomenon of protection against a distal tumor, at the same time as the parental tumor is progressing (13). A recent reexploration of concomitant immunity suggests that regulatory T cells are the major players in this phenomenon (14).

Our data can also be interpreted in the light of our current understanding of immunologic memory. Because we have previously shown that the antitumor effector response in the murine model of mesothelioma depends on CD8 T cells (2), we now propose a model of antitumor immunity that is based on the ability of CD8 T cells to persist in the absence of tumor antigen. Conventional CD8 T-cell memory exists in the absence of antigen and is maintained by the homeostatic cytokines IL-7 and IL-15 (15). It seems most likely that this type of memory is responsible for maintenance of long-term antitumor immunity in our experiments. However, recent data show that CD8 T-cell memory that develops in the *presence of persisting antigen*, as is the case with progressive tumor growth, is different: such memory cells depend on the continued presence of antigen for survival and do not respond to homeostatic cytokines (16, 17). Applied to our tumor model, it can be predicted that during the progressive phase of growth, antigen-dependent memory will dominate and that surgery functions as an "immunologic reset." In the case of partial resection combined with gemcitabine and FGK-45, we hypothesize that this reset is enabled by (i) removal of the bulk of the tumor by surgery (thus reducing tumor-driven immunosuppression) followed by (ii) increased antigen presentation in the draining lymph node resulting from gemcitabine treatment (7) and (iii) licensing of dendritic cells by CD40 stimulation (18). Combined, this favors a *de novo* CD8 T-cell

response, which is fuelled by gemcitabine-induced antigen cross-presentation in the post-surgery inflammatory environment. Because antigen is only transiently present, this response should facilitate the formation of appropriate memory.

In the case of complete resection (group 5, Table 1), we assume that the (decaying) level of post-surgery antigen cross-presentation is sufficient to prime a new response, whereas the tumor burden remains low or nonexistent. Chemotherapy alters this, potentially by both killing the remaining tumor cells (thereby enhancing antigen presentation and decreasing tumor burden) *and* killing lymphocytes. The most vulnerable lymphocytes will include the rapidly dividing CD8 T cells that constitute a post-surgery effector response. Thus, without the benefit of added antigen presentation (i.e., after complete resection), chemotherapy after surgery has the potential to damage the ensuing immune response. Under these circumstances, FGK-45 treatment would not be expected to promote memory, as there would be no effector cells in the system. As far as we are aware, there have been no clinical trials to date using humanized activating anti-CD40 antibodies, although several groups are currently pursuing this strategy. Adams et al. recently trialed a partially agonistic chimeric anti-CD40 in primates at doses up to 100 mg/kg without reported incidence (19). For comparison, the dose of activating anti-CD40 in our mouse experiments is equivalent to 5 mg/kg. Hopefully, results from

preclinical studies in mice, such as ours, will serve to guide the dosing regimen in clinical trials once humanized activating anti-CD40 becomes available.

In conclusion, our results are consistent with a model in which development of an effective antitumor immune response is critically dependent on the deviation of the default induction of antigen-dependent memory to an antigen-independent memory. We propose that surgery creates the opportunity to reset the antitumor response and that combination chemoimmunotherapy protocols could provide the tools to achieve this. The clinical implication of this is that when complete resection is a realistic option, chemocombination/immunocombination therapy will be more effective when combined with some form of vaccination. If, in contrast, some tumor remains *in situ*, combination therapy has the potential to create long-lasting immunity against any such recurrence.

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