

# Synergistic Anti-Tumor Responses After Administration of Agonistic Antibodies to CD40 and IL-2: Coordination of Dendritic and CD8<sup>+</sup> Cell Responses<sup>1</sup>

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In cancer, the coordinate engagement of professional APC and Ag-specific cell-mediated effector cells may be vital for the induction of effective antitumor responses. We speculated that the enhanced differentiation and function of dendritic cells through CD40 engagement combined with IL-2 administration to stimulate T cell expansion would act coordinately to enhance the adaptive immune response against cancer. In mice bearing orthotopic metastatic renal cell carcinoma, only the combination of an agonist Ab to CD40 and IL-2, but neither agent administered alone, induced complete regression of metastatic tumor and specific immunity to subsequent rechallenge in the majority of treated mice. The combination of anti-CD40 and IL-2 resulted in significant increases in dendritic cell and CD8<sup>+</sup> T cell number in advanced tumor-bearing mice compared with either agent administered singly. The antitumor effects of anti-CD40 and IL-2 were found to be dependent on CD8<sup>+</sup> T cells, IFN- $\gamma$ , IL-12 p40, and Fas ligand. CD40 stimulation and IL-2 may therefore be of use to promote antitumor responses in advanced metastatic cancer. *The Journal of Immunology*, 2003, 170: 2727–2733.

CD40, a member of the TNF receptor superfamily, plays a critical role in dendritic cell development and serves to costimulate T and B cell responses (1–3). The ligand for CD40 is present on activated T and NK cells (4). CD40-CD40 ligand interactions are critical for optimal T cell responses, and the impairment of these interactions fosters a tolerogenic state (5–7). CD40 stimulation is also capable of inducing production of numerous inflammatory cytokines by monocytes and dendritic cells (8, 9). Thus, CD40 represents an important molecular target for initiating and/or amplifying nascent immune responses against tumors. It has become increasingly apparent that single agents that usually target defined compartments of the immune system often fail to exert potent and sustained immunomodulatory effects, particularly in instances of advanced disease. This may be due in part to the lack of coordinate enhancement of other immune compo-

nents that are needed for the complex, interdependent interactions that are required for productive immune responses. In that regard, both CD40 stimulation (10, 11) and cytokine therapy with IL-2 (12) have been shown to exert some antitumor effects in various mouse tumor models. Further, although IL-2 alone may induce potent antitumor responses against renal cell cancer and melanoma in humans (13, 14), its overall utility may be limited by a narrow therapeutic window. We hypothesized that the combination of CD40 stimulation and IL-2 may provide a novel, well-tolerated means to induce immune responses against cancer. Our results show, in two tumor models, that CD40 stimulation and IL-2 synergize to induce complete regression of metastatic tumors in vivo due to potentiation of dendritic and T cell responses.

## Materials and Methods

### Mice

BALB/c, C57BL/6, and C.B.-17 SCID mice were obtained from the Animal Production Area of the National Cancer Institute (Frederick, MD). BALB/c CD40<sup>-/-</sup> and C57BL/6 CD4<sup>-/-</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c IFN- $\gamma$ <sup>-/-</sup>, IL-12 p40<sup>-/-</sup>, perforin<sup>-/-</sup>, and *gld* mice were maintained in our own pathogen-free breeding colony (National Cancer Institute). All mice were between 8 and 10 wk of age. Animal care was provided in accordance with the procedures outlined in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication 86-23, 1985).

### Tumor cell lines

Renca, a BALB/c renal cell adenocarcinoma, was maintained in mice by serial in vivo passage (15). SIRCC-1.2, a moderately differentiated BALB/c renal cell adenocarcinoma, C57BL/6-derived Lewis lung carcinoma (3LL), and BALB/c-derived A20 B cell lymphoma cells were grown as previously described (16–18).

### Reagents

Recombinant human IL-2 was provided by Chiron Corp. (Emeryville, CA). Agonist rat anti-mouse CD40 (clone FGK115) (19) or purified rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was administered (100  $\mu$ g i.p.) daily, five times per week for 2 wk, for a total of 10 injections, and IL-2 was given in twice daily injections (300,000 IU i.p.).

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twice a week for a total of eight injections. IFN- $\gamma$  was purchased from PeproTech (Rocky Hill, NJ).

#### *In vivo tumor models*

One hundred thousand Renca cells were injected into the kidneys of BALB/c mice. The primary tumor was removed by unilateral nephrectomy 11 days post-tumor cell injection as previously described (15). Treatment was initiated the day following resection of the primary tumor. Some mice were rechallenged s.c. with  $10^5$  Renca cells by injection into the flank of surviving mice or with  $10^5$  SIRCC-1.2 cells. One million 3LL cells were administered by s.c. injection into the flank of C57BL/6 mice. Tumor volume was measured biweekly. Some mice were rechallenged with  $10^6$  3LL s.c. in the contralateral flank. All tumor survival experiments contained 10–15 mice/treatment group.

#### *In vivo depletion experiments*

CD8<sup>+</sup> cells were depleted *in vivo* by i.p. injection of rat anti-mouse CD8 (clone 19-178). Two doses of Ab (163  $\mu$ g/dose) were administered before the beginning of therapy and were continued three times weekly during the course of immunotherapy (>90% depletion). Control animals received 10% normal rat serum. NK cells were depleted *in vivo* by i.p. injection of rabbit anti-asialo-GM1 (10  $\mu$ l in 90  $\mu$ l of PBS/dose; Wako Chemicals, Richmond, VA). Ab was administered 4 days before the beginning of and on the first day of immunotherapy (>75% depletion in splenic DX5<sup>+</sup> cells). Control animals received 10% normal rabbit serum.

#### *Flow cytometric analysis*

Leukocytes were labeled as previously described (15). Briefly,  $10^6$  cells were labeled with Ab. Cells stained with biotinylated Abs were then incubated with streptavidin-Red 670 (Life Technologies, Gaithersburg, MD). Samples were analyzed on a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Anti-CD8 mAb (clone 53-6.7; BD PharMingen, San Diego, CA)-labeled cells in the lymphocyte gate were identified as CD8<sup>+</sup> T cells. Dendritic cells were identified with mAbs to CD11c (clone HL3; BD PharMingen) and MHC class II (clone AMS-321). Nonspecific binding was corrected with isotype-matched controls.

#### *CTL induction from Renca-bearing mice*

Splenocytes were isolated from BALB/c mice after *in vivo* therapy. Responder splenocytes ( $4\text{--}5 \times 10^6$ ) were cultured with  $1.5 \times 10^5$  IFN- $\gamma$  (200 U/ml) pretreated and irradiated (100 Gy) Renca stimulator cells in 24-well plates with complete RPMI medium supplemented with  $1 \times$  nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES,  $5 \times 10^{-5}$  M 2-ME, and 10 U/ml IL-2.

#### *Tumor-specific IFN- $\gamma$ production*

Splenocytes were stimulated with Renca *in vitro* as described for CTL induction. Cells ( $10^5$ ) were then incubated with  $10^4$  Renca or A20 stimulator cells (effector:stimulator ratio, 10:1) or medium alone in 96-well plates at 37°C for 16 h. For the blocking experiments, 10  $\mu$ g/ml anti-CD8 mAb (53-6.7; BD PharMingen) or anti-CD4 mAb (GK1.5; BD PharMingen) was added to the culture. Cell-free supernatants were harvested, and IFN- $\gamma$  was measured by ELISA (R&D Systems, Minneapolis, MN).

#### *IFN- $\gamma$ ELISPOT assay*

Millipore 96-well, flat-bottom plates (Millipore, Bedford, MA) were coated with anti-mouse IFN- $\gamma$  mAb (BD PharMingen) and blocked with RPMI 1640 containing 10% FCS. Effector cells ( $10^4$ /well) and  $2 \times 10^4$  target (Renca) cells were added to triplicate wells. The negative control included effector cells in the absence of target cells, target cells in the absence of effector cells, and medium alone. Cells were incubated overnight and then lysed. The addition of biotinylated anti-mouse IFN- $\gamma$  Ab (BD PharMingen) was followed by alkaline phosphatase-streptavidin conjugate. The enzyme substrate was 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich, St. Louis, MO). The plates/membranes were air-dried overnight before image analysis on a series I ImmunoSpot analyzer (Cellular Technology, Cleveland, OH). The results were expressed as a number of spots per  $10^4$  effector cells after negative controls were subtracted.

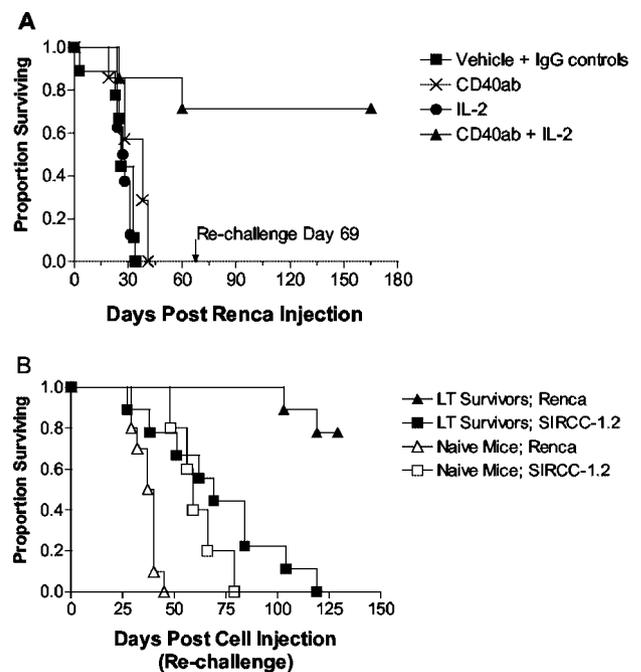
## Results

### *Anti-CD40 and IL-2 synergize for regression of established metastatic disease*

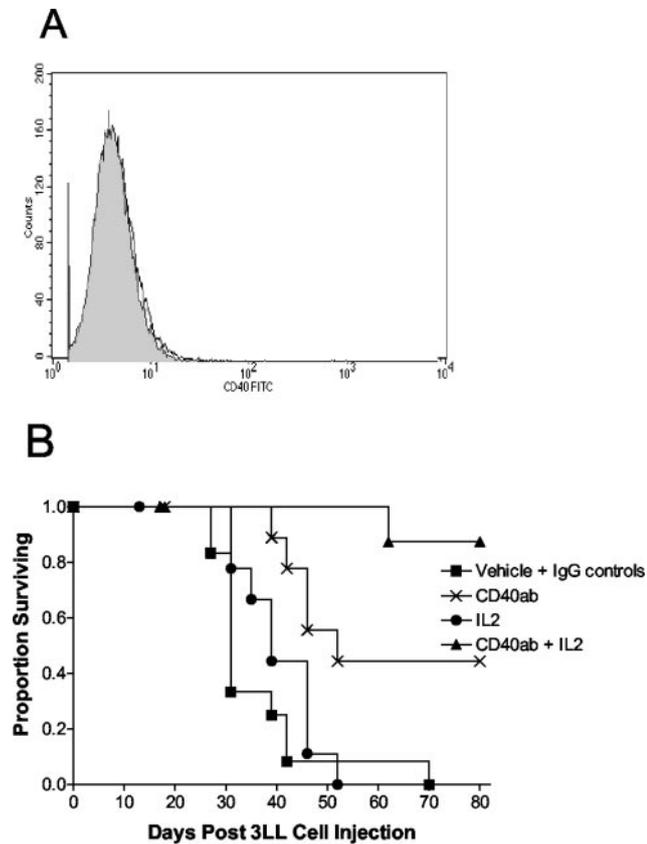
CD40-specific agonistic mAbs can exert antitumor effects *in vivo* (10, 11), while IL-2 is a Federal Drug Administration-approved

therapy for renal cell carcinoma (RCC)<sup>3</sup> and melanoma. We employed the orthotopic Renca RCC model where tumor cells are injected intrarenally into syngeneic BALB/c mice, and progressive growth is accompanied by the formation of lung and/or liver metastases in 100% of the mice (15, 20). Eleven days after tumor implantation, a unilateral nephrectomy of the tumor-bearing kidney was performed to remove the primary tumor, and the residual metastatic disease was treated using agonistic anti-CD40 and IL-2. Anti-CD40 was administered (100  $\mu$ g i.p.) daily, five times per week for 2 wk, and IL-2 was given in twice daily injections (300,000 IU i.p.) twice a week. The results demonstrate that both IL-2 and anti-CD40 administered as single agents by these same regimens failed to significantly prolong the survival of tumor-bearing mice (Fig. 1A). In contrast, the administration of both anti-CD40 and IL-2 resulted in a marked and highly significant ( $p < 0.005$ ) prolongation of survival, with >70% of the mice remaining disease-free for >160 days. When the surviving mice were rechallenged with Renca after 69 days, 80–100% demonstrated complete resistance to the original tumor (Fig. 1A), but no resistance to challenge with another syngeneic RCC (Fig. 1B). Subsequent dose-response studies in mice demonstrated that even doses of

<sup>3</sup> Abbreviation used in this paper: RCC, renal cell carcinoma.



**FIGURE 1.** Anti-CD40 and IL-2 provide antitumor effects in tumor-bearing mice. *A*, Following surgical removal of the primary tumor, the Renca-bearing BALB/c mice ( $n = 8\text{--}9$ /group) were treated with anti-CD40 (×) or recombinant human IL-2 (●), singly or in combination (▲), or were given vehicle as controls (■). The combination of anti-CD40 and IL-2 resulted in significantly ( $p < 0.005$ ) greater survival compared with control mice or mice receiving anti-CD40 or IL-2 alone. Effects on survival were noted. Long term (LT) survivors (>69 days) were then rechallenged with Renca cells *i.v.* Representative data from one of five similar experiments are shown. *B*, Naive mice (△ and □) or mice that received Renca tumor, as described above, and the combination of anti-CD40 and IL-2 therapy (■ and ▲; day 76 following initial tumor challenge) were challenged with SIRCC-1.2 (□ and ■) or Renca (△ and ▲) via s.c. injection in the flank. Significant increases in survival were observed only in rechallenged recipients of Renca ( $p < 0.0001$ ). Survival analysis was plotted according to the Kaplan-Meier method, and statistical differences were determined with the log-rank test.



**FIGURE 2.** The effects of anti-CD40 and IL-2 on a CD40<sup>-</sup> tumor line. *A*, The 3LL Lewis lung carcinoma was examined for CD40 expression by flow cytometry and was found to be negative even after IFN- $\gamma$  incubation (100 U for 16 h). *B*, The 3LL tumor-bearing mice ( $n = 10$ /group) were treated with anti-CD40 ( $\times$ ) and recombinant human IL-2 ( $\bullet$ ), singly or in combination ( $\blacktriangle$ ), or were given vehicle as controls ( $\blacksquare$ ). The mice were monitored for survival. The combination of anti-CD40 and IL-2 resulted in a significant increase in survival compared with controls or mice given IL-2 alone (by log-rank test,  $p < 0.0001$ ) or anti-CD40 alone ( $p < 0.05$ ) animals. Representative data from one of two similar experiments are shown.

anti-CD40 up to 250  $\mu$ g/injection given in the absence of IL-2 did not produce any protective effect in this advanced tumor model (data not shown), further demonstrating the dramatic nature of the synergy with IL-2. These results show that anti-CD40 and IL-2 can synergize to induce complete regression of advanced Renca RCC metastases in the lungs and liver of mice, and that this response is associated with the development of tumor-specific immunity upon rechallenge.

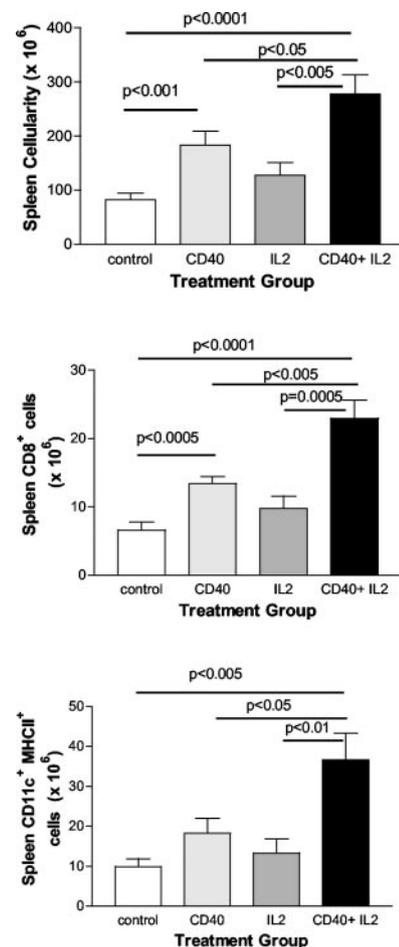
*Therapeutic synergy between anti-CD40 and IL-2 is dependent on stimulation of a host response and independent of CD40 expression on the tumor cells*

Because CD40 has been demonstrated to be present on numerous normal and neoplastic cells, including RCCs (21), the expression of CD40 on Renca tumor cells was assessed. We found that these cells constitutively express low, but detectable, levels of CD40 that could be further augmented by incubation with IFN- $\gamma$  (data not shown). To determine whether the therapeutic synergy between anti-CD40 and IL-2 required direct binding of anti-CD40 to the tumor cells or was solely dependent on stimulation of the host response, we tested the ability of anti-CD40 and IL-2 to treat CD40<sup>-</sup> 3LL Lewis lung carcinoma. Of note, 3LL cells are constitutively negative for CD40 expression, and no induction was

observed even after pretreatment with IFN- $\gamma$  (Fig. 2*A*). C57BL/6 mice were injected s.c. with 3LL cells, and 3 days later the mice were treated with our standard regimen of anti-CD40 and IL-2. The results demonstrate that the combination of anti-CD40 and IL-2 resulted in significant increases ( $p < 0.05$ ) in survival in mice bearing these established CD40<sup>-</sup> tumors compared with either treatment alone (Fig. 2*B*), although with this tumor, anti-CD40 alone did provide some protection. These results demonstrate that the combination of anti-CD40 and IL-2 induces complete regression of established tumors in different strains of mice, and antitumor effects can occur in the absence of tumor cell CD40 expression.

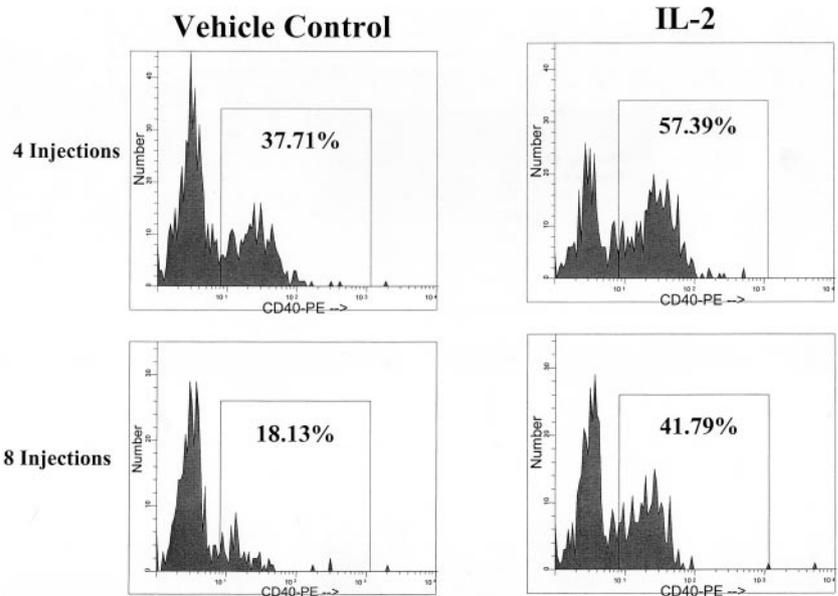
*Coordinate engagement of dendritic cell and T cell compartments by anti-CD40 and IL-2 treatment*

The nonessential role for tumor-associated CD40 expression on 3LL tumor cells, in the acute effects of anti-CD40 and IL-2 supported our initial hypothesis that anti-CD40 would enhance the number and function of the professional APC compartment, while IL-2 would expand the activated T cell compartment. To formally test this hypothesis, we examined splenic and hepatic leukocytes from Renca-bearing mice treated with anti-CD40, IL-2, or anti-CD40 and IL-2. Mice were assessed 2–3 days after treatment. The



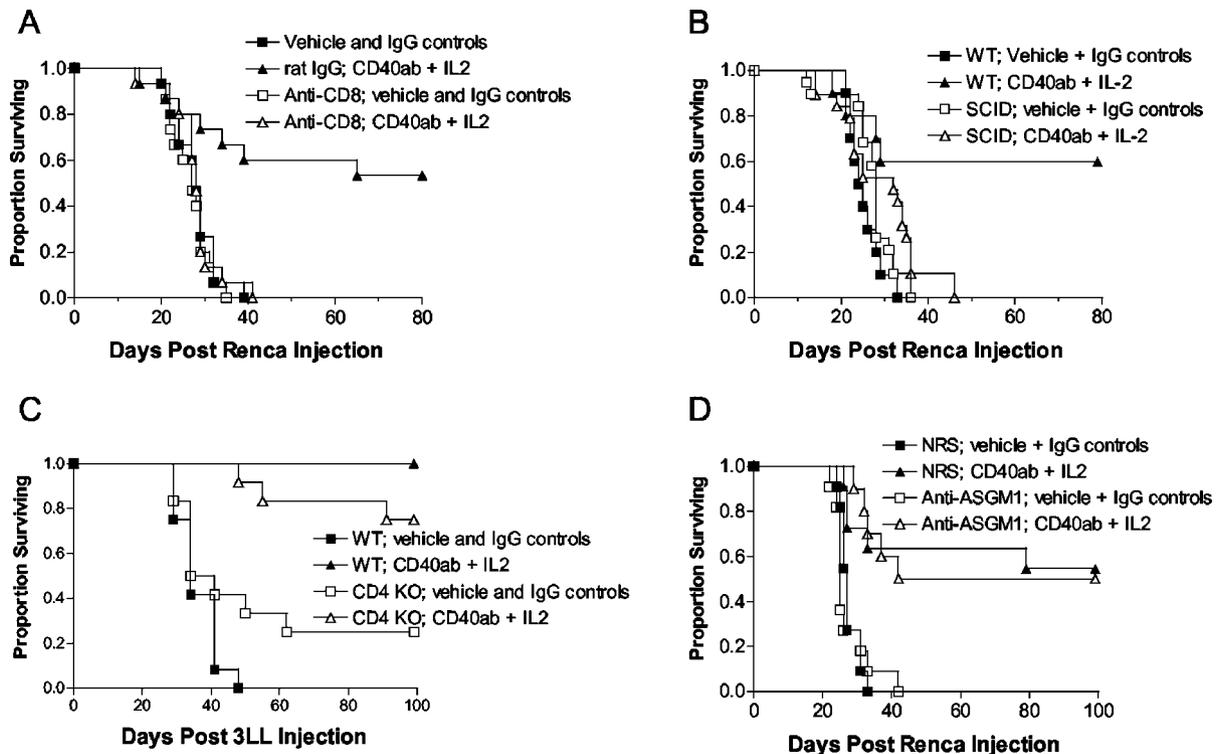
**FIGURE 3.** Immune parameters following treatment with CD40 and IL-2 in Renca-bearing mice. Two or three days following completion of therapy, mice were analyzed for the effect of treatment on CD8<sup>+</sup> T cell and dendritic cells in the spleen. Two or three mice were analyzed per group in each experiment. The results are a compilation of seven independent experiments. Statistical differences are indicated (by Student's  $t$  test,  $p < 0.05$ ).

**FIGURE 4.** IL-2 administration enhances CD40 expression on splenic dendritic cells. Representative histograms depict CD40 expression on CD11c<sup>+</sup> MHCII<sup>+</sup> cells in nontumor-bearing mice that received IL-2 or vehicle control, twice daily, twice a week for 1 or 2 wk. Splenic dendritic cells were analyzed on the day following the fourth and eighth injections of IL-2.



results show a significant increase in the numbers of splenic leukocytes (Fig. 3, *top panel*;  $p < 0.05$ ), CD8<sup>+</sup> T cells (Fig. 3, *middle panel*;  $p < 0.005$ ), and MHC II<sup>+</sup>, CD11c<sup>+</sup> dendritic cells (Fig. 3, *bottom panel*;  $p < 0.05$ ) in mice receiving the combination of anti-CD40 and IL-2 compared with either control mice or those

treated with anti-CD40 or IL-2 alone. To determine the mechanism by which the combination of IL-2 and anti-CD40 may increase dendritic cell formation and/or function, we assessed the effects of IL-2 alone on CD40 expression on dendritic cells, reasoning that cytokines induced after IL-2 may act on dendritic cells, making



**FIGURE 5.** Requirement for CD8<sup>+</sup> cells for antitumor activity induced by anti-CD40 and IL-2 treatment. BALB/c mice received intrarenal injections of Renca, followed 11 days later by nephrectomy and the standard course of treatment with anti-CD40 and IL-2 for 12 days (as described in *Materials and Methods*;  $n = 9-13$  mice/group; A, B, and D). Survival analysis was plotted according to the Kaplan-Meier method, and statistical differences were determined with the log-rank test. Representative data from one of two similar experiments are presented for each panel. A, Anti-CD40 and IL-2 treatment in CD8<sup>+</sup> cell-replete ( $\blacktriangle$ ) and CD8<sup>+</sup> cell-depleted ( $\triangle$ ) mice. Treatment with anti-CD8 (as described in *Materials and Methods*) completely abrogated the protective effects of anti-CD40 and IL-2 ( $p < 0.001$ ). B, Effect of anti-CD40 and IL-2 in mice with SCID. C, B17 SCID mice received vehicle control injections ( $\square$ ) or anti-CD40 and IL-2 ( $\triangle$ ). No difference in survival was observed. D, C57BL/6 CD4<sup>+/+</sup> and CD4<sup>-/-</sup> mice received s.c. injection of 3LL tumor cells 3 days before the initiation of CD40 and IL-2 treatment or control injections. Mice were monitored for tumor growth, and animals with large or necrotic tumors were euthanized. D, Anti-CD40 and IL-2 treatment or control injections in NK cell-replete and NK cell-depleted mice. Treatment with anti-ASGM1 (as described in *Materials and Methods*) did not modify the therapeutic effect of CD40 and IL-2 treatment.

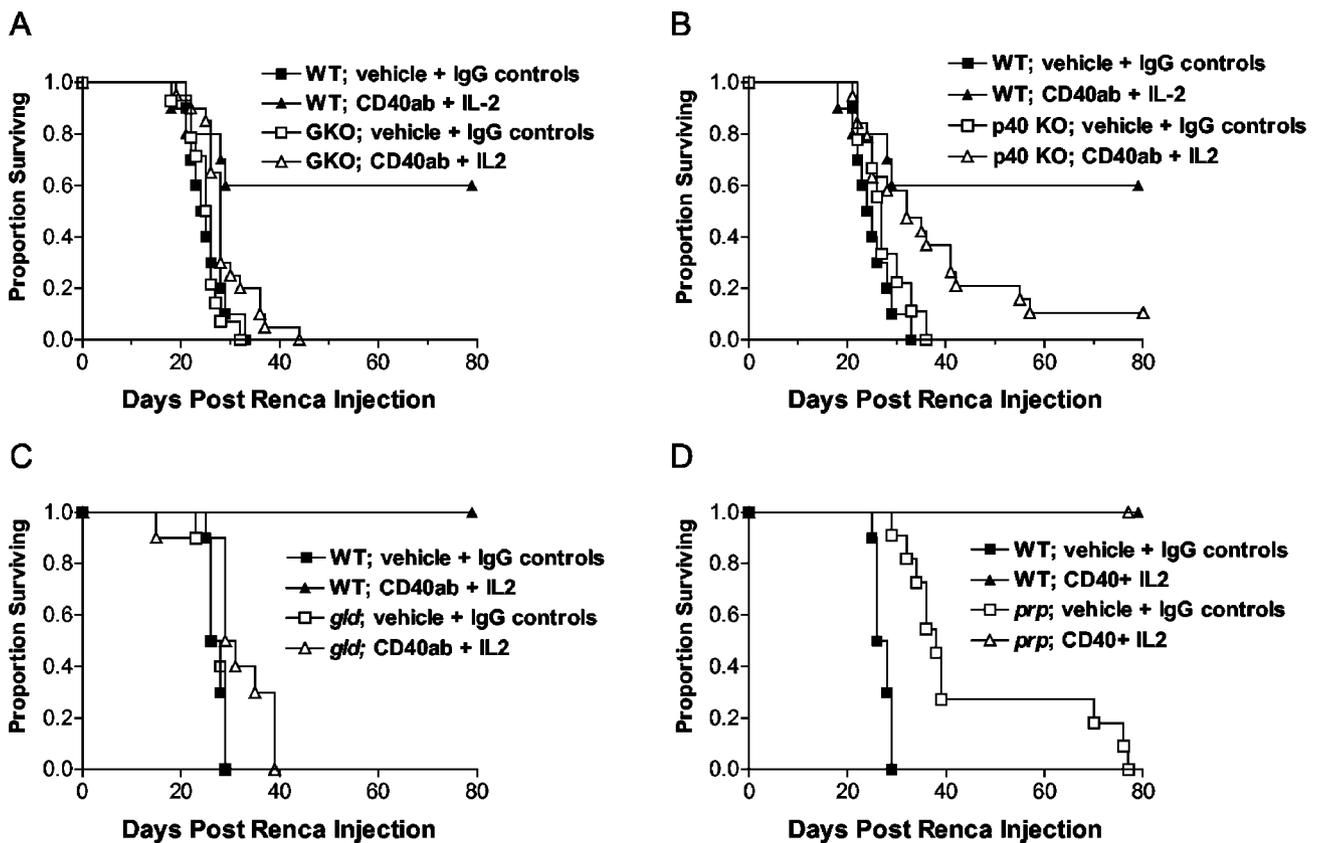
them more receptive to anti-CD40 stimulation. The results demonstrate that the administration of IL-2 alone (300,000 IU, twice daily, twice a week for 2 wk) to mice was capable of augmenting CD40 expression on dendritic cells (Fig. 4), thereby making the cells more responsive to CD40 stimulation. This at least partially may account for the synergy of anti-CD40 and IL-2 in the promotion of dendritic cell generation.

To determine whether CD8<sup>+</sup> T cells were required for the regression of tumors observed after anti-CD40 and IL-2 treatment, CD8<sup>+</sup> T cells were depleted *in vivo* before and during treatment. The results show that depletion of CD8<sup>+</sup> cells eliminated the therapeutic activity of anti-CD40 and IL-2 (Fig. 5A). A similar loss of anti-CD40 plus IL-2 efficacy was observed in Renca-bearing mice with SCID, which lack T and B cells, but have normal NK cell activity (Fig. 5B). In contrast, CD40 and IL-2 treatment was effective in C57BL/6 CD4<sup>-/-</sup> mice bearing syngeneic 3LL tumors (Fig. 5C), demonstrating in this tumor model that CD4<sup>+</sup> cells are not a critical mediator of the antitumor activity of this therapy. These results demonstrate that CD8<sup>+</sup> T cell are pivotal for the protection seen after anti-CD40 and IL-2 treatment, and the lack of protection in tumor-bearing SCID mice suggests that NK cells may not play a dominant role. The lack of a contribution by NK cells was also demonstrated through studies in which Renca (Fig. 5D) or 3LL-bearing mice (data not shown) were specifically depleted of NK cells with no diminution of the therapeutic efficacy of anti-CD40 and IL-2.

The coordinate amplification of the dendritic and T cell compartments and the dependency on CD8<sup>+</sup> T cells suggest the potent engagement of a beneficial Th1 (IFN- $\gamma$ , IL-12) adaptive response by anti-CD40 and IL-2. The dependency of the complete responses induced by anti-CD40 and IL-2 on these cytokines was demonstrated by a loss of therapeutic efficacy in both tumor-bearing IFN- $\gamma$ <sup>-/-</sup> and IL-12 p40<sup>-/-</sup> mice (Figs. 6, A and B). Interestingly, serum IFN- $\gamma$  levels in mice were negligible following anti-CD40 and IL-2 treatment (<11 pg/ml), suggesting that focused production of IFN- $\gamma$  at local sites of the immune response was occurring. The antitumor effects were also found to be dependent on Fas ligand, but not perforin, as protection from combination therapy was lost when Fas ligand-deficient mice were used as recipients (Fig. 6C). Perforin-deficient recipient mice were as capable as wild-type mice in yielding potent antitumor responses after combination therapy (Fig. 6D). These results indicate that CD40 agonist Ab and IL-2 therapy was capable of augmenting CD8<sup>+</sup> T cells and Th1-type responses to the tumor.

#### Enhanced tumor-specific, CD8<sup>+</sup> T cell-mediated responses after anti-CD40 and IL-2 treatment

As CD8<sup>+</sup> T cells were critical for the protection seen following anti-CD40 and IL-2 (Fig. 5A), and mice were resistant to rechallenge (Fig. 1B), we assessed the tumor specificity of splenic CD8<sup>+</sup> T cells recovered from the tumor-bearing mice following treatment. Analysis of Renca-specific CTL activity, as determined by



**FIGURE 6.** Requirement of IFN- $\gamma$  and Fas ligand for the antitumor activity induced by anti-CD40 and IL-2 treatment. **A**, Effects of anti-CD40 and IL-2 using IFN- $\gamma$ <sup>-/-</sup> mice as recipients. BALB/c IFN- $\gamma$ <sup>-/-</sup> mice received vehicle control injections (□) or anti-CD40 and IL-2 (△). The combination of anti-CD40 and IL-2 had no protective effects in IFN- $\gamma$ <sup>-/-</sup> mice compared with control mice. **B**, Effects of anti-CD40 and IL-2 in IL-12 p40<sup>-/-</sup> mice. BALB/c IL-12 p40<sup>-/-</sup> mice received vehicle control injections (□) or anti-CD40 and IL-2 (△). The protective effects of anti-CD40 and IL-2 treatment were significantly diminished compared with the effects of their combination in wild-type BALB/c mice ( $p < 0.05$ ). **C**, Fas is a mediator of anti-CD40 with IL-2 therapy. BALB/c wild-type (▲) and *gld*<sup>-/-</sup> (△) mice received anti-CD40 and IL-2 therapy. The combination of anti-CD40 and IL-2 had no protective effect in *gld* mice compared with wild-type mice ( $p < 0.0001$ ). **D**, BALB/c wild-type (▲) and perforin (*prp*)<sup>-/-</sup> (△) mice received anti-CD40 and IL-2 therapy. Anti-CD40 and IL-2 treatment was effective in both wild-type and perforin-deficient mice.

Table I. Effects of anti-CD40 agonist Ab stimulation and IL-2 treatment in vivo on the ability of the recovered T cells to produce IFN- $\gamma$  in response to Renca ex vivo<sup>a</sup>

In Vivo Therapy	Mouse	IFN- $\gamma$ (pg/ml)				ELISPOT/10 <sup>4</sup> Cells	
		Renca	Renca/anti-CD4	Renca/anti-CD8	Effector alone	Renca	A20
Control	1, 2, 3	1310	1056	<2	203	0	–
	4, 5	377	141	<2	17		
IL-2	1	447	443	<2	12	6	–
	2	347	281	<2	123		
Anti-CD40	1	470	457	19	18	15	–
	2	319	291	<2	20		
	3	823	762	<2	42		
IL-2/anti-CD40	1	6426	6199	1227	274	31	–
	2	2458	2465	350	76		
	3	1025	776	283	100		

<sup>a</sup> Renca-bearing mice were treated with anti-CD40 agonist Abs and/or IL-2. After the last day of treatment, spleens from mice were obtained and CTL were generated in a 5-day culture as described in *Materials and Methods*. Spleens from individual animals were analyzed separately except in the control group where, due to lower cell yields, splenocytes were pooled. After 5 days, tumor-specific responses were measured by coinubation of splenocytes with Renca or A20 tumor cells with or without blocking Abs to anti-CD8 or anti-CD4 for 16 h. IFN- $\gamma$  levels in culture supernatants were determined by ELISA. In vitro-stimulated spleen cells were pooled from individual animals in the IFN- $\gamma$  ELISPOT assay.

cytotoxicity, was not significantly different between treatment groups following completion of immunotherapy (data not shown); however, CD8<sup>+</sup> T cells from the mice receiving anti-CD40 and IL-2 produced appreciable IFN- $\gamma$  specifically in response to Renca and not the unrelated A20 (H2<sup>d</sup>) tumor (Table I). CD4<sup>+</sup> T cells did not contribute to the tumor-specific IFN- $\gamma$  production response. Ag specificity was further demonstrated by ELISPOT assay, which revealed increased IFN- $\gamma$  production in response to Renca, but not A20, indicating that heightened responses to the tumor by T cells occurred in mice receiving the combination therapy (Table I). These results demonstrate that increases in tumor-specific CD8 responses are induced after combination treatment. The increase in IFN- $\gamma$  production detected in vitro is supported by the critical role of IFN- $\gamma$  in the in vivo therapy model (Fig. 6A). These results combined with the results from in vivo depletion and cytokine knockout studies demonstrate that anti-CD40 and IL-2 coordinately enhance the number and/or function of dendritic cells and CD8<sup>+</sup> T cells in association with potent therapeutic effects in mice bearing advanced cancer.

## Discussion

Immunotherapy offers great promise for the successful eradication of cancer. However, it is apparent that effective strategies for amplification of immune responses are required due to the weakly immunogenic nature of most human cancers. This report demonstrates that the synergistic activity of anti-CD40 agonist Abs and IL-2 exerts potent anti-tumor effects in mice. This protection occurs coincident with dendritic and CD8<sup>+</sup> T cell expansion and increased function. Previously, anti-CD40 agonist Ab stimulation has been shown to exert pleiotropic effects on immune cell function and differentiation. CD40 stimulation has been demonstrated to induce dendritic cell differentiation, induce cytokine production by monocytes, affect T cell help, and augment NK cell function in vivo (8, 11). We speculate that one of the mechanisms underlying the increased antitumor effects seen in the CD4 knockout recipients is due to the ability of anti-CD40 to bypass T cell help as reported by other investigators (22, 23). In addition, CD40 stimulation also has been shown to induce IL-12 production by dendritic cells (2), leading us to speculate that the loss of efficacy by the combined treatment using IL-12 knockout mice may be due in part to the production of IL-12 by dendritic cells after treatment. It is of interest that modest immunomodulatory effects and minimal

to no antitumor effects were detected in mice treated with either agent singly. This may be due in part to the advanced tumor burden and extensive metastases present in the model used for our studies. The amount of anti-CD40 needed in our studies to show synergy with IL-2 was much less than the doses reported in previous studies, in which anti-CD40 administration alone showed some protective effects (11, 24, 25). These results suggest that the combination of anti-CD40 and IL-2 might be even more effective in those models in which anti-CD40 alone showed efficacy.

A recent report demonstrated that anti-CD40 administration resulted in the accelerated depletion of CD8<sup>+</sup> T cells in tumor-bearing mice (26). This report speculated that other cytokines may be required in addition to CD40 stimulation to incur sustained responses and is in agreement with the results presented in our study. It is also of interest that little toxicity was observed in our studies. Cytokine regimens have been associated with significant toxicities, in part due to the large amounts needed to be given systemically for the appropriate cells to respond. The use of CD40 stimulation may provide a sensitizing role, in that a cascade may result with the local production of cytokines at the tumor site and allow for further amplification with subsequent IL-2 administration. This is supported by the data demonstrating that IFN- $\gamma$  appears to play a critical role in the protective effects, but little IFN- $\gamma$  is detected in the serum after treatment. This local response may also account for the minimal toxicities of this regimen vs systemic administration of other cytokine combinations. It would be of interest to assess the effects of other cytokines (i.e., IL-7, IL-12, and IL-15) either with this combination or with anti-CD40 alone to ascertain whether greater or more sustained antitumor effects can be achieved. It will also be of interest to determine whether this combination can be used in infectious disease models, as both CD40 and IL-2 have been shown to play a role in immune responses to various pathogenic organisms (27, 28).

It appears that in this model that IL-2 is doing much more than solely promoting T cell survival. The data demonstrating increases in CD40 expression on dendritic cells after IL-2 administration represents a novel means by which IL-2 can synergize with CD40 stimulation to start an immunomodulatory cascade. A role for IL-2 activity on dendritic cells has been postulated based on recent studies suggesting that the production of IL-2 by dendritic cells may serve a critical role in priming of naive T cells and act to regulate dendritic cells in an autocrine manner (29, 30).

Previous studies with IL-12 and IL-2 treatment for advanced metastatic renal cell cancer in mice demonstrated the importance of the IL-2 pulse regimen to reduce toxicity. Both CD40 agonist Ab with IL-2 (Fig. 1A) and IL-12 and IL-2 treatment (15) regimens result in 60–100% long term survivors following the establishment of orthotopic Renca tumors. Both treatment regimens are dependent on IFN- $\gamma$  and Fas/Fas ligand for therapeutic efficacy (Fig. 6) (20); however, the presence of CD40 on numerous neoplastic cell types suggests that it can also exert direct antitumor effects due to activation-induced cell death (31). It is possible that greater antitumor responses are obtained when CD40 is present on the tumor, although it is not required to generate potent antitumor responses after treatment. The generation of CD40 dominant negative Renca cell lines should shed light on this question. Additionally, the lack of involvement of NK cells in our models does not preclude them from playing a role in other tumor systems. Indeed, the addition of IL-2 may increase the activation-induced cell death potential of NK cells when CD40 is present on the cancer cell. However, the data presented here clearly demonstrate that the synergy in inducing tumor-specific T cells is the predominant mechanism underlying the successful eradication of the cancer. These results then suggest that CD40 stimulation with agonistic CD40 Abs and IL-2 provides potent immunomodulatory effects in vivo and may be of use in advanced cancer.

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