Biology of Blood and Marrow Transplantation 8:316-325 (2002)

Administration of Either Anti-CD40 or Interleukin-12 following Lethal Total Body Irradiation Induces Acute Lethal Toxicity Affecting the Gut

Julie A. Hixon,[1] Miriam R. Anver,[2] Bruce R. Blazar,[3] Angela Panoskaltsis-Mortari,[3] Robert H. Wiltrout,[4] William J. Murphy[1]

[1]SAIC-Frederick, [2]Pathology Histotechnology Laboratory, SAIC-Frederick, NCI-Frederick, Frederick, Maryland; [3]Department of Pediatrics, Division of Bone Marrow Transplantation, University of Minnesota Cancer Center, Minneapolis, Minnesota; [4]Laboratory of Experimental Immunology, NCI-Frederick, Frederick, Maryland

Correspondence and reprint requests: William J. Murphy, SAIC-Frederick, NCI-Frederick, Bldg 567, Rm 210, Frederick, MD 21702 (e-mail: murphyw@mail.ncifcrf.gov).

Received January 30, 2002; accepted April 15, 2002

ABSTRACT

Interleukin (IL)-12 and antibodies against CD40 have demonstrated antitumor effects in a variety of in vivo model systems. However, both agents can also mediate significant toxicities either when used following lethal TBI or when administered in combination with other agents such as IL-2. In this study, we assessed the effects of anti-CD40 monoclonal antibody (MoAb) and IL-12 in lethally irradiated mice. Acute lethal toxicity was observed following the administration of either 10 μg anti-CD40 MoAb (FGK45) or 0.5 μg of recombinant murine (rm)IL-12 that resulted in 100% mortality of all mice within 4 to 6 days. Histological evaluation revealed destruction of the normal gut architecture in both anti-CD40 MoAb– and rmIL-12–treated mice. Analysis of serum cytokine levels in the lethally irradiated mice receiving anti-CD40 MoAb demonstrated a marked increase of interferon (IFN)-γ and IL-12 p40, whereas mice receiving rmIL-12 demonstrated a marked increase of IFN-γ. Lethally irradiated IL-12 p40 knock-out mice were resistant to anti-CD40–induced toxicity, suggesting that the lack of IL-12 p40 with no possibility of making functional IL-12 p70 is key for this toxic reaction. Similarly, lethally irradiated IFN-γ knock-out mice were completely resistant to rmIL-12–induced toxicity, suggesting that IFN-γ is a major player in IL-12–mediated toxicity. These results suggest that both anti-CD40 MoAb and rmIL-12 induce an acute fatal toxicity characterized by similar intestinal pathology and mediated in part by IFN-γ.

KEY WORDS

Antibodies • Transplantation • Cytokines • In vivo animal models

INTRODUCTION

CD40 is a 48-kd transmembrane protein that is a member of the nerve growth factor/tumor necrosis factor (TNF) receptor superfamily [1,2]. Members of the TNF receptor superfamily are structurally homologous and function to regulate cell proliferation, differentiation, and death using shared signal transduction pathways [3]. Although originally described as playing a role in humoral immune responses, it is now known that CD40 plays a wider role in regulating immune function by increasing both costimulatory molecules and antigen presentation [4,5]. CD40 also contributes to the inflammatory process by inducing the secretion of inflammatory cytokines including interleukin (IL)-1, IL-6, IL-12, and TNF-α [3].

CD40 is expressed on a variety of cell types including monocytes, dendritic cells, endothelial cells, and carcinomas [3]. The expression of CD40 on a variety of carcinomas including those of the bladder, kidney, ovary, skin, and breast and the role of CD40 in the promotion of immune function makes CD40 an attractive target for immunotherapy [6,7]. We and others have demonstrated both in vitro and in vivo antitumor activity following CD40 stimulation using both lymphomas [8-10] and solid-tissue carcinomas [11,12]. Although CD40 stimulation occurs without any

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adverse effects in the nonirradiated animal, we have previously reported an acute morbidity that affected the intestine following CD40 stimulation in lethally irradiated mice [13]. The intestinal lesions and rate of morbidity observed in our CD40-mediated toxicity models are very similar to those observed following the administration of IL-12 with IL-2 in nonirradiated mice.

IL-12 is an important proinflammatory cytokine that stimulates the production of IFN-γ from natural killer (NK) and T-cells and serves as a key initiator of cell-mediated immune responses [14,15]. In addition, IL-12 has demonstrated potent antiangiogenic and antitumor effects when used alone [16,17] or in combination with IL-2 [18,19] that are dependent on the production of IFN-γ [20]. Although proinflammatory cytokines such as IL-12 and IL-2 play a role in the immunotherapy of neoplastic disease, their use can be complicated by the induction of lethal cytokine-induced shock affecting the intestine and lungs [21]. Interestingly, the rate of morbidity and the intestinal lesions in both the IL-2/IL-12 and the anti-CD40 monoclonal antibody (MoAb) model systems are surprisingly similar. Both demonstrated rapid morbidity, with all mice succumbing within 4 to 6 days from elevated levels of proinflammatory cytokines and the destruction of normal gut architecture. Although the toxicity of the administration of IL-12 in combination with IL-2 has been evaluated [21], the role of IL-12 administration following lethal total body irradiation (TBI) in mice has been evaluated using only C57BL/6 mice [22]. Irradiation, either alone or in combination with chemotherapy, is commonly used in conventional cancer therapy to reduce the tumor burden of the host. Changes in cytokine profiles following irradiation therapy could result in unexpected toxicity when cytokines are used to promote efficient host-antitumor immune responses. In the current study, we evaluate the effects of IL-12 using mice that have received TBI, and extend our findings concerning the acute lethal toxicity observed following CD40 stimulation in lethally irradiated mice to further define the mechanism of CD40-mediated toxicity. We also present data that we have published previously [13] as a comparison to the IL-12 data, because the studies had been performed side by side.

MATERIALS AND METHODS

Mice

Female BALB/c and male and female BALB/c IFN-γ or IL-12 p40 knock-out mice were obtained from the Animal Production Area (National Cancer Institute [NCI]-Frederick, Frederick, MD) and were not used until they were 8 weeks of age. Animals were cared for humanely according to both the US Public Health Policy on the Care and Use of Animals and the Guide for the Care and Use of Laboratory Animals. NCI-Frederick facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Antibodies and Recombinant Murine IL-12

Anti–mouse CD40 (FGK45, rat immunoglobulin G1 agonistic MoAb) was produced as ascites as described previously [8]. The antibody was at a concentration of 9.7 mg/mL, kept frozen until use, and diluted in phosphate buffered saline (PBS) for injections. Recombinant murine (rm)IL-12 was purchased from Peprotech (Nutley, NJ). The cytokine was reconstituted to a concentration of 1 mg/mL in PBS, stored at –70°C until use, and diluted in PBS for injections.

Bone Marrow Transplantation

Recipient BALB/c, BALB/c IFN-γ, or BALB/c IL-12 p40 knock-out mice were exposed to a ^{137}Cesium source at a dose rate of 212 cGy/minute and received 750 cGy TBI. Mice were then given 10^6 syngeneic bone marrow cells (BMC) intravenously (IV). Additional studies were performed to evaluate the role of irradiation in rmIL-12–induced toxicity. For these studies, mice were exposed to either 800 cGy, 700 cGy, or 600 cGy TBI, and given 10^6 syngeneic BMC IV. Immediately following irradiation and transplantation in both model systems, the mice received daily intraperitoneal (IP) injections of either murine agonistic monoclonal CD40, clone FGK45 (10 μg), rmIL-12 (0.5 μg), or PBS control in 0.2 mL. Injections were begun immediately following irradiation and transplantation and continued daily for 4 to 5 days. All experiments were assayed with 6 to 10 mice per group and were performed 2 to 6 times with a representative experiment being shown.

Histology

Selected organs including small intestine, colon, lungs, and liver were fixed in 10% formalin. Gut was collected in a "swiss roll" for microscopic examination of maximal surface area. Tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Slides were evaluated microscopically by a veterinary pathologist without reference to the various treatment groups. Grading was as follows: 1, minimal; 2, mild; 3, moderate; 4, severe; M, missing; P, present; –, no significant lesion. The experiments were performed 5 times with a representative being shown.

Serum Cytokine Determination

For determination of serum IFN-γ and IL-12 p40 levels, blood was collected 48 hours after initial injection of either agonistic anti-CD40 MoAb, rmIL-12, or PBS control. Samples were assayed by enzyme-linked immunosorbent assay (ELISA) using a Quantikine M mouse IFN-γ Immunoassay or IL-12 p40 Kits (R&D Systems, Minneapolis, MN). The assay was performed according to instructions from the manufacturer.

RESULTS

Administration of Either Anti-CD40 MoAb or rmIL-12 Induces Rapid Toxicity in Lethally Irradiated Mice

We have previously demonstrated acute toxicity characterized by the destruction of gut tissue following CD40 stimulation via MoAb in lethally irradiated mice [13]. Similar acute mortality and intestinal lesions have been demonstrated in nonirradiated SCID mice treated with rmIL-12 in combination with IL-2 [21]. Although morbidity was not observed with IL-12 alone, mild intestinal lesions occurred in the nonirradiated SCID mice, and these lesions were exacerbated by the addition of IL-2. Therefore, we examined the administration of rmIL-12 using our lethal TBI model in BALB/c (750 cGy), C57BL/6 (950 cGy), and CB.17 SCID (350 cGy) mice and compared the results to

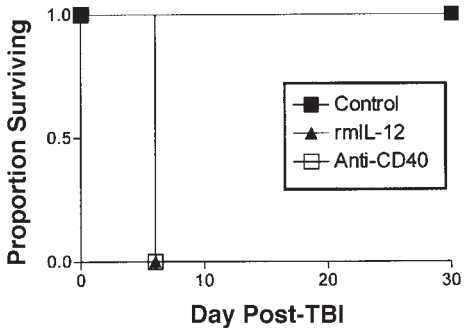

Figure 1. Administration of either anti-CD40 MoAb (FGK45) or rmIL-12 after lethal TBI induces acute lethal toxicity. BALB/c mice were given 750 cGy TBI and syngeneic BMT followed by daily IP injections of PBS, 10 μg anti-CD40 MoAb, or 0.5 μg of rmIL-12 for 5 days. All mice receiving either anti-CD40 MoAb or rmIL-12 demonstrated significant ($P < .0005$) mortality by day 6, whereas 100% of mice receiving control sera survived. Experiments were performed 5 times with similar results and used 6 to 10 mice per group.

the acute toxicity observed following administration of anti-CD40 MoAb. To evaluate the effects of both rmIL-12 and anti-CD40 MoAb following TBI, syngeneic BMC were transferred into lethally irradiated (750 cGy) recipients, and the mice were given either 10 μg anti-CD40 MoAb (FGK45), 0.5 μg rmIL-12, or PBS control for 4 to 5 days, beginning the day of irradiation and transplantation. All BALB/c mice receiving either anti-CD40 MoAb or rmIL-12 died within 4 days after lethal TBI, whereas all control mice survived (Figure 1). Similar results were observed in lethally irradiated (950 cGy) C57BL/6 mice given anti-CD40 MoAb and in lethally irradiated (350 cGy) CB17.SCID mice treated with either anti-CD40 MoAb or rmIL-12 (data not shown). It is of interest to note that although the lethally irradiated (950 cGy) C57BL/6 mice succumbed following anti-CD40 MoAb administration, they required a dose of anti-CD40 MoAb that was 10 times greater than that given to lethally irradiated (750 cGy) BALB/c mice to demonstrate the same effect. In addition, lethally irradiated (950 cGy) C57/BL6 mice were completely resistant to up to 1.0 μg of rmIL-12. Taken together, these data suggest that there may be strain differences involved in anti-CD40 MoAb– and rmIL-12–mediated toxicity. Severe intestinal lesions, which were similar in both treated groups (Table 1), were the most likely cause of mortality in all affected strains receiving either anti-CD40 or rmIL-12. The lungs and livers of the mice were found on histological evaluation to be normal. The most extensive damage occurred in the villi of the small intestine (Figure 2). Compared to the control mice, treated mice had villi that were markedly blunted, and many of the shortened villi were fused together. Villous epithelium had variable cytoplasmic vacuolation and diffuse karyomegaly. The lamina propria had an infiltrate of mononuclear cells and neutrophils. Both control and treated mice had mildly hyperplastic crypts. Lesions were also present in the colons of mice receiving anti-CD40 MoAb or rmIL-12 (Figure 3). The principal change was diffuse goblet cell depletion, with foci of sloughed cells in the crypt lumens. Some of the affected crypts were mildly hyperplastic. These results demonstrate that administration of either anti-CD40 MoAb or rmIL-12 following lethal TBI induces rapid morbidity in several different strains of mice that is characterized by the formation of significant gut lesions that are not observed in control mice, but the liver and lung were unaffected.

Reduction of TBI Eliminates rmIL-12–Induced Toxicity

We have previously demonstrated the elimination of anti-CD40–induced toxicity using sublethally irradiated mouse models [13]. To assess the role of TBI in rmIL-12–induced toxicity, recipient BALB/c mice were given either lethal or sublethal doses of irradiation, followed by the transfer of syngeneic BMC and daily injections of rmIL-12 beginning at day 0 and continuing for 5 days. Administration of rmIL-12 following 800 cGy TBI resulted in rapid mortality, with 100% of mice dying by day 6, whereas mice irradiated with 700 cGy TBI demonstrated 60% mortality

Table 1. *Effect of Administration of Anti-CD40 and rmIL-12 on Gut following Lethal Total Body Irradiation**

	PBS	Anti-CD40	Anti-CD40	rmIL-12	rmIL-12
Small Intestine					
Villous blunting	–	4MF	4MF	4MF	3MF
Villous fusion	–	4MF	4MF	4MF	3MF
Karyomegaly	–	2MF	–	2MF	1MF
Colon					
Goblet cell depletion	–	3MF	3MF	3MF	4MF
Crypt cell hyperplasia	–	1MF	1MF	2MF	2MF
Lymphoid depletion	–	–	4	–	4
Sloughed cells, crypt lumen	–	3MF	3MF	2MF	–
Lungs	N	N	N	N	N
Liver					
Inflammation	–	2	–	–	–

*BALB/c mice were lethally irradiated, given a syngeneic BMT, and were given daily intraperitoneal injections of either PBS, 10 μg of anti-CD40 MoAb, or 0.5 μg rmIL-12 for 4 days. Mice were killed for histological evaluation 24 hours after the last intraperitoneal injection. Lesions were evaluated and scored as follows: 1, minimal; 2, mild; 3, moderate; 4, severe, MF, multifocal; –, no significant lesion; N, normal. Experiments were performed 5 times with similar results. Data represent individual mice.

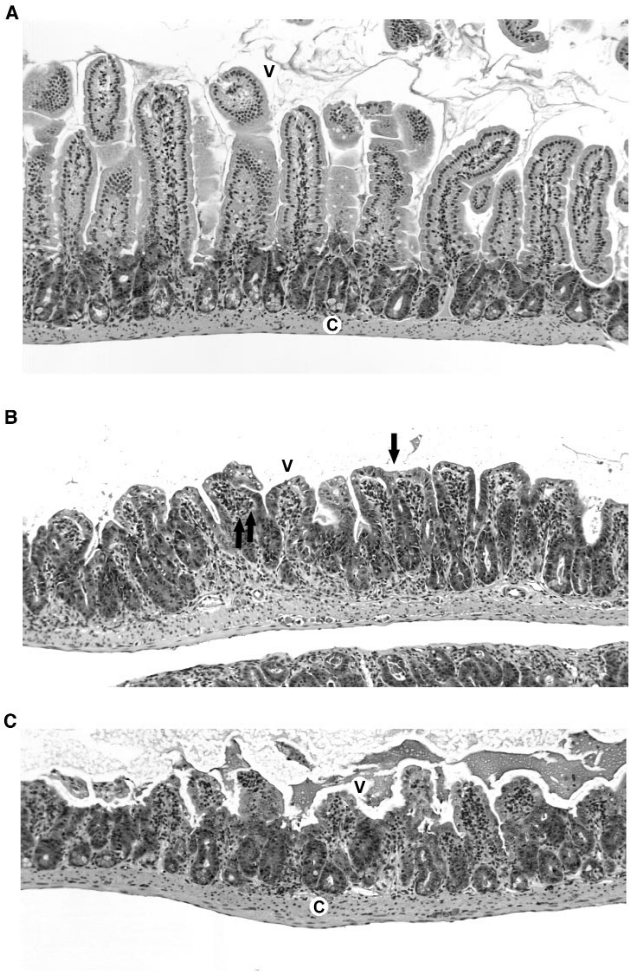

Figure 2. Anti-CD40 MoAb and rmIL-12 given after lethal TBI mediate the destruction of small intestine. BALB/c mice received 750 cGy TBI and syngeneic BMT followed by daily IP injections of 10 μg of anti-CD40 MoAb, 0.5 μg rmIL-12, or control serum for 5 days. Mice underwent complete necropsy 24 hours following the last IP injection. A, BALB/c mice treated with control serum. Small intestine has normal-length villi (V) and mildly hyperplastic crypts (C). B, BALB/c mice treated with anti-CD40 MoAb. Villi (V) are extremely blunted; several are fused (arrow). Cellular infiltrate in lamina propria (double arrow). Mildly hyperplastic crypts (C). C, BALB/c mice treated with rmIL-12. Blunted villi (V) similar to those observed with anti-CD40 MoAb treatment; hyperplastic crypts (C). Experiments were performed 5 times with 1 to 3 mice undergoing histological evaluation in each experiment. Hematoxylin and eosin, original magnification ×10.

at day 6 (Figure 4). Similar to sublethally irradiated anti-CD40–treated mice [13], 100% of mice receiving 600 cGy TBI and rmIL-12 survived (Figure 4). Histological analysis revealed a reduction in the severity of the lesions in the guts of sublethally irradiated mice compared with that of mice receiving lethal doses of irradiation in response to rmIL-12 administration (data not shown). Thus, similar to the diminished mortality and pathology observed following CD40 stimulation in sublethally irradiated mice, the severity of rmIL-12–induced toxicity is also dependent on the intensity of conditioning.

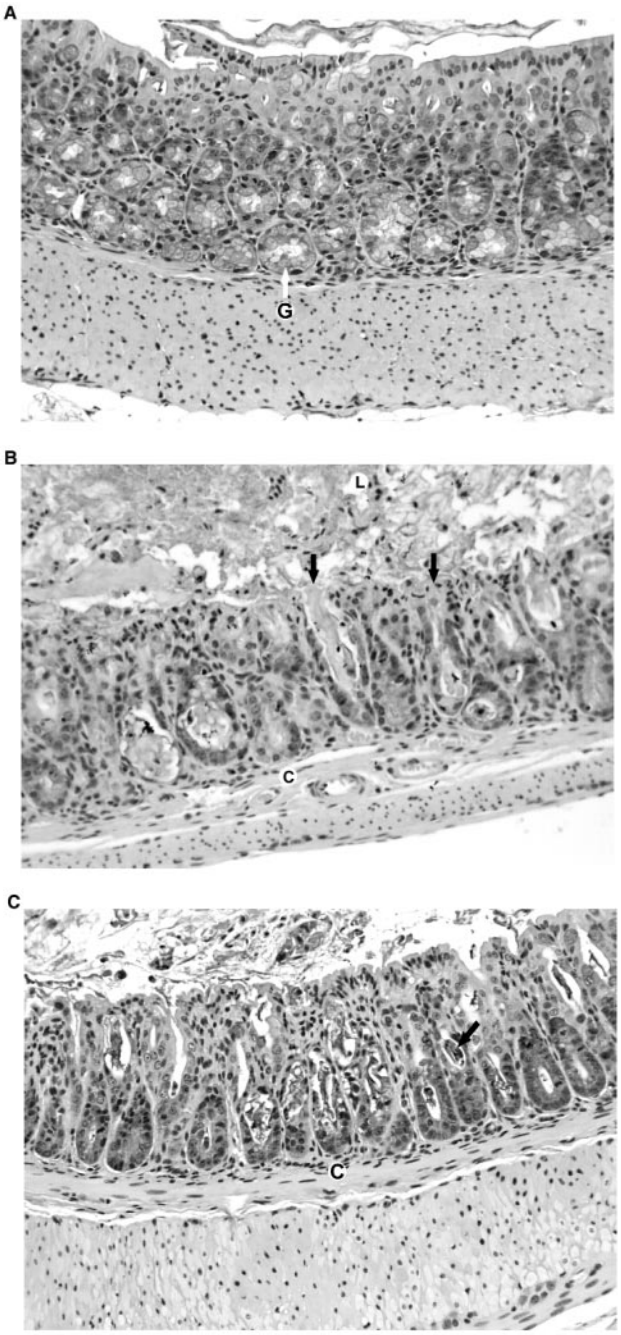

Figure 3. Anti-CD40 MoAb and rmIL-12 given after lethal TBI mediates goblet cell depletion in the crypts of the colon. BALB/c mice received 750 cGy TBI and syngeneic BMT followed by daily IP injections of 10 μg of anti-CD40 MoAb, 0.5 μg rmIL-12, or PBS control for 5 days. Mice underwent complete necropsy 24 hours following the last IP injection. A, BALB/c mice treated with control serum. Many goblet cells (G) are present in the crypts of the colon. B, BALB/c mice treated with anti-CD40 MoAb. Goblet cells in crypts (C) are depleted of mucus, which is present in crypt lumens (arrow) and streaming into the bow lumen (L). C, BALB/c mice treated with rmIL-12. Goblet cell depletion in crypts (C) similar to that observed with anti-CD40 MoAb treatment; lumens with mucus and some sloughed cells (arrow). Mild crypt hyperplasia. Experiments were performed 5 times with 1 to 3 mice undergoing histological evaluation in each experiment. Hematoxylin and eosin, original magnification ×20.

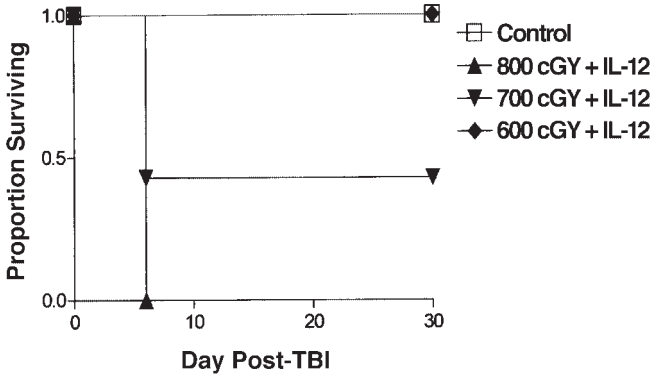

Figure 4. Decreased TBI eliminates rmIL-12–induced toxicity. BALB/c mice were irradiated with either 800 cGy, 700 cGy, or 600 cGy TBI, given a syngeneic BMT, and injected daily IP with 0.5 μg of rmIL-12 or PBS control. All mice receiving 800 cGy and 60% of mice receiving 700 cGy succumbed by day 6. All mice receiving 600 cGy demonstrated significant (P = .0003) survival compared to mice receiving 800 cGy. Experiments were performed 1 time with 10 mice per group.

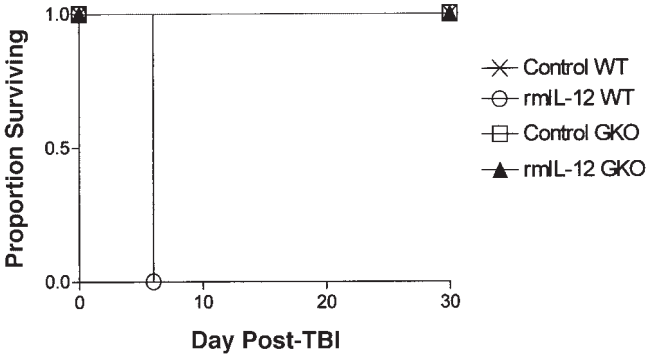

Figure 5. Absence of IFN-γ improves survival in lethally irradiated rmIL-12–treated mice. BALB/c WT or GKO mice were lethally irradiated and given a syngeneic BMT and daily IP injections for 5 days of either 0.5 μg of rmIL-12 or PBS control. Mice deficient in IFN-γ and treated with rmIL-12 showed significantly ($P \leq .0001$) increased survival compared to similarly treated WT mice.

Administration of Either rmIL-12 or Anti-CD40 MoAb Increases IFN-γ Levels in the Serum of Lethally Irradiated Mice

We have previously demonstrated an increase of IFN-γ in the serum of lethally irradiated mice following CD40 stimulation [13]. Therefore, the role of IFN-γ in rmIL-12–induced toxicity was evaluated. Recipient BALB/c mice received 750 cGy TBI, followed by the transfer of syngeneic BMC. The mice then received daily IP injections of 10 μg anti-CD40 MoAb (FGK45), 0.5 μg rmIL-12, or PBS control. Serum was collected from the mice following 4 injections of anti-CD40 MoAb and rmIL-12, and IFN-γ levels were assessed by ELISA. Results from these experiments demonstrated a marked and significant ($P \leq .05$) increase in the level of IFN-γ in the serum of lethally irradiated mice receiving either anti-CD40 MoAb or rmIL-12 compared to that of control mice (Table 2). These results suggest that IFN-γ may play a role in both the anti-CD40 MoAb– and rmIL-12–induced toxicities observed in lethally irradiated mice.

Absence of IFN-γ Eliminates rmIL-12–Induced Toxicity

We have previously demonstrated a reduction in the toxicity associated with anti-CD40 MoAb using IFN-γ knock-out mice [13]. Given the high levels of IFN-γ in the serum of lethally irradiated mice receiving rmIL-12, we postulated that removal of IFN-γ would also reduce rmIL-12–induced toxicity. To address the role of IFN-γ in rmIL-12–induced toxicity, lethally irradiated BALB/c IFN-γ knock-out recipients were given syngeneic BMC from IFN-γ knock-out donors, and wild-type BALB/c control recipients were given syngeneic BMC from wild-type BALB/c donors. Mice then received either 0.5 μg rmIL-12 or PBS control for 5 days. All of the wild-type mice receiving rmIL-12 succumbed by day 6, whereas 100% of the IFN-γ–deficient mice receiving rmIL-12 survived (Figure 5). Histological evaluation of IFN-γ–deficient mice receiving rmIL-12 demonstrated a dramatic lessening of the severity of intestinal lesions including complete elimination of villi blunting and fusion in the small intestine (Table 3; Figure 6B) and marked reduction of the goblet cell depletion in the colon (Figure 6D) compared to that in similarly treated wild-type mice (Figure 2C and 3C). We have previously demonstrated diminished lesions in the small intestine and colon of IFN-γ–deficient mice receiving anti-CD40 MoAb [13]. Thus the mortality and gut lesions observed following administration of rmIL-12 and anti-CD40 MoAb are due, in part, to the production of IFN-γ.

Table 2. *Serum IFN-γ Levels Increase after Lethal TBI and Administration of Either Anti-CD40 MoAb or rmIL-12**

Conditions	IFN-γ Level in Serum, pg/mL
Control WT	<9.4 ± 0.07
Anti-CD40 WT	645.5 ± 6.4†
rmIL-12 WT	2712.5 ± 870†

*Lethally irradiated BALB/c wild-type (WT) mice were bled for serum 48 hours after the initial injection of either 10 μg of anti-CD40 MoAb, 0.5 μg rmIL-12, or PBS control and assayed by enzyme-linked immunosorbent assay. Data are the mean ± SD of 3 mice. Experiments were performed twice with similar results.

†Significantly different ($P \leq .05$) from PBS-treated control.

Administration of Anti-CD40 MoAb to Lethally Irradiated Mice Increases IL-12 Levels in the Serum

We have previously demonstrated the production of IFN-γ following CD40 stimulation in lethally irradiated mice [13]. IFN-γ knock-out mice, however, were afforded only partial protection from anti-CD40–induced toxicity, leading us to speculate that additional factors may be involved in the mortality and destruction of normal gut architecture observed following lethal TBI and CD40 stimulation. Given the similarities in rate of mortality and pathological changes observed in both rmIL-12– and anti-CD40 MoAb–induced toxicities, we next evaluated the role of IL-12 in anti-CD40–induced toxicity. Lethally irradiated (750 cGy) BALB/c mice received syngeneic BMC followed

Table 3. *Absence of IFN-γ Diminishes rmIL-12–Induced Gut Damage**

Small Intestine	WT Ctl	WT Ctl	WT Ctl	WT IL-12	WT IL-12	WT IL-12	WT Ctl	GKO Ctl	GKO Ctl	GKO IL-12	GKO IL-12	GKO IL-12
Villous blunting	1MF	–	–	4MF	4MF	4MF	–	–	–	1MF	–	–
Villous fusion	–	–	–	3MF	4MF	4MF	–	1MF	1MF	–	–	–
Vacuolation, villi	–	–	–	3MF	3MF	3MF	–	–	–	–	–	–

*BALB/c WT and IFN-γ knock-out (GKO) mice received lethal TBI and a syngeneic BMT, followed by daily intraperitoneal injections of 0.5 μg rmIL-12 or PBS control (Ctl) for 5 days. Mice were histologically evaluated 24 hours after the last intraperitoneal injection. Lesions were evaluated and scored as follows: 1, minimal; 2, mild; 3, moderate; 4, severe; MF, multifocal; –, no significant lesion. Experiments were performed twice with similar results. Data represent individual mice.

by daily IP injections of either 10 μg anti-CD40 MoAb or PBS control. Mice were bled for serum at day 2, and IL-12 p40 and p70 levels were assessed by ELISA. Results from these experiments demonstrated a marked and significant ($P \leq .001$) increase in the level of IL-12 p40 in the serum of lethally irradiated mice receiving anti-CD40 MoAb compared to that of control mice (Table 2). A similar increase in levels of IL-12 p70 was observed in lethally irradiated mice receiving anti-CD40 MoAb (data not shown). These data suggest that in addition to IFN-γ, IL-12 p40 plays a role in the morbidity of lethally irradiated mice treated with anti-CD40 MoAb.

Absence of IL-12 p40 Eliminates Anti-CD40–Induced Toxicity

The high levels of serum IL-12 p40 in lethally irradiated mice receiving anti-CD40 MoAb led to an investigation into the role of IL-12 in anti-CD40–induced toxicity. Lethally irradiated BALB/c p40 knock-out mice were given syngeneic BMC from BALB/c p40 knock-out donors, and wild-type BALB/c control mice were given syngeneic BMC from wild-type BALB/c donors. Mice were then treated daily for 4 days with either 10 μg anti-CD40 MoAb or PBS control. Interestingly, all wild-type mice receiving anti-CD40 MoAb following lethal TBI succumbed by day 4, whereas 100% of

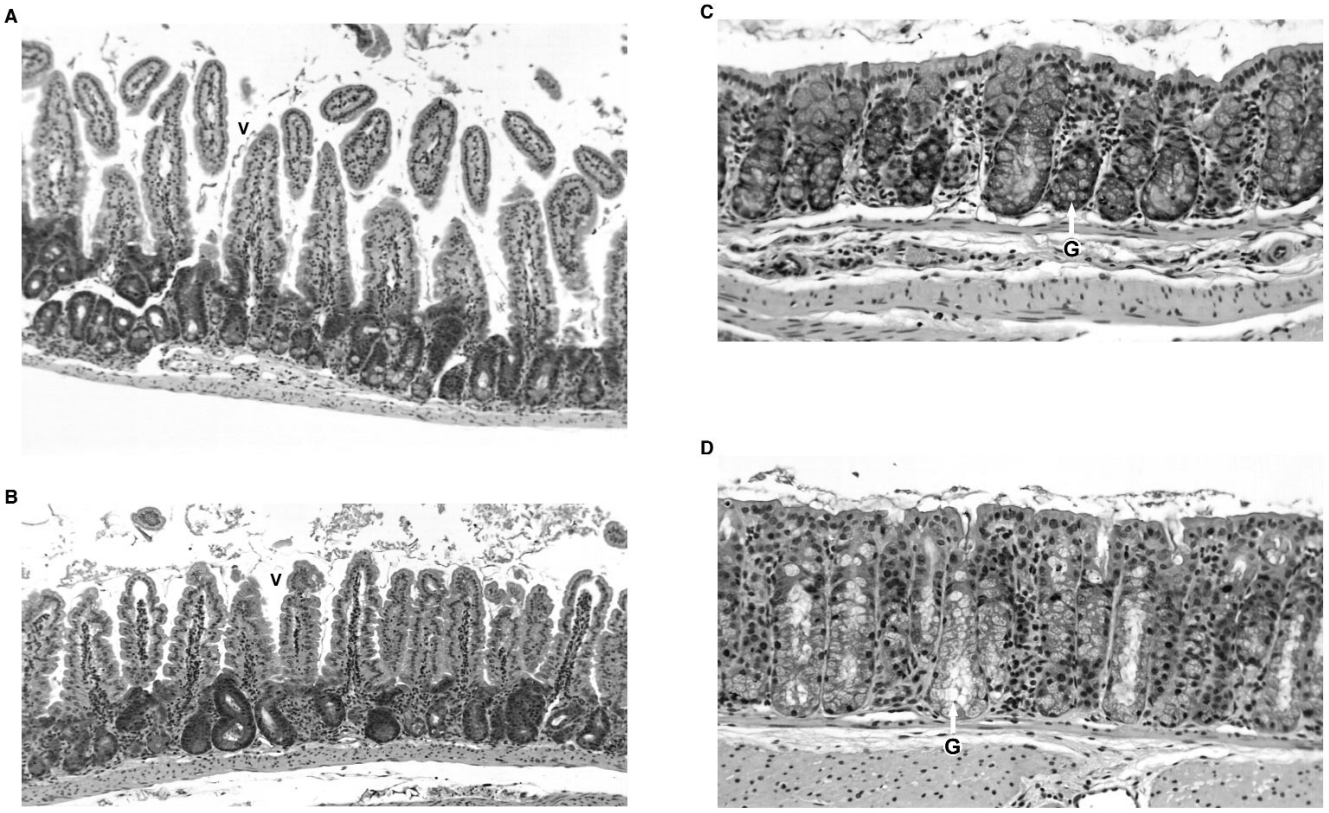

Figure 6. Elimination of IFN-γ protects small intestine and colon from rmIL-12–mediated destruction. A and C, BALB/c GKO mice treated with control serum. Small intestine (6A) has normal-length villi (V) and colon (6C) has crypts with normal populations of goblet cells (G). B and D, BALB/c GKO mice treated with rmIL-12. Small intestine (6B) has normal-length villi (V), and there is a lessening of goblet cell (G) depletion in the crypts of the colon (6D) compared to that of similarly treated WT mice (Figures 2 and 3). Experiments were performed 2 times with 1 to 3 mice undergoing histological evaluation in each experiment. Hematoxylin and eosin, original magnification ×10.

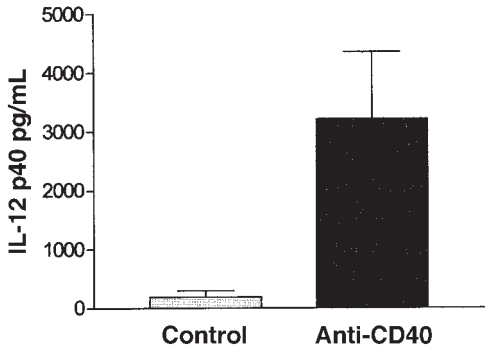

Figure 7. Administration of anti-CD40 MoAb to lethally irradiated mice increases IL-12 levels in serum. BALB/c mice received 750 cGy TBI and a syngeneic BMT, followed by daily IP injections of 10 μg of anti-CD40 MoAb or PBS control. Mice were bled for serum after 3 injections, and the serum was analyzed by ELISA. Mice given anti-CD40 MoAb demonstrated significantly elevated ($P \leq .001$) serum IL-12 levels compared to similarly treated control mice. Data are presented as a combination of 2 experiments with 2 to 3 mice per group.

the IL-12 p40 knock-out mice survived (Figure 8). Histological evaluation of the mice revealed a marked reduction of the villi blunting and fusion in the small intestine (Figure 9B) and complete elimination of goblet cell depletion in the colon (Figure 9D) in the IL-12 p40 knock-out mice compared to that of similarly treated wild-type control mice, demonstrating destruction of normal gut architecture (Figure 2B and 3B). Thus IL-12 is a key mediator of anti-CD40–induced mortality and intestinal destruction.

DISCUSSION

We have previously demonstrated a fatal cytokine-induced disease affecting the intestine following lethal TBI and CD40 stimulation. In this study, we have extended these findings as well as demonstrated a rapid and fatal toxicity affecting the intestine following lethal TBI and the administration of rmIL-12 that occurs in several different strains of mice. Interestingly, both the CD40- and IL-12–toxicity model systems have similar kinetics with all treated mice succumbing within 4 days, with the exception of C57BL/6 mice, which were completely resistant to up to 1.0 μg rmIL-12. In addition, histological evaluation revealed identical lesions in the guts of mice treated with either anti-CD40 MoAb or rmIL-12. Similar to the results obtained with the anti-CD40 MoAb toxicity system, decreasing the amount of conditioning either reduced or eliminated rmIL-12–induced toxicity. Not only does this finding demonstrate the importance of conditioning, it further illustrates the similarities between the anti-CD40 MoAb– and rmIL-12–induced toxicities. Given these similarities, we speculated that the same mediator was responsible for the observed mortality and pathology. Because we have previously demonstrated a role for IFN-γ in anti-CD40–induced lethality, we evaluated for the presence of IFN-γ using our IL-12 toxicity system.

Administration of rmIL-12 following lethal TBI resulted in dramatic increases of serum IFN-γ levels compared to those observed in control mice. IL-12 induces production of IFN-γ from NK and T-cells, and a role for IFN-γ in the antitumor and antiangiogenic effects of IL-12 has been demonstrated [20]. IFN-γ is also involved in the activation of macrophages and primes them for the secretion of other proinflammatory cytokines such as IL-1, IL-6, IL-12, and IL-18 [23]. Although the production of proinflammatory cytokines is important for early resistance to intracellular infections [15], these cytokines are also thought to be involved in pathogenesis resulting in tissue injury to the gut that is mediated by monocytes/macrophages and NK cells [24]. Our data demonstrating the complete elimination of morbidity and pathology following the administration of rmIL-12 to IFN-γ knock-out mice further support the role of IFN-γ as a mediator of tissue injury to the gut.

Although we have previously demonstrated a role for IFN-γ in anti-CD40–induced toxicity [13], it is possible that other proinflammatory cytokines such as endotoxin, IL-18, IL-23, TNF, IL-2, or IL-12 may be involved in mediating the observed pathology. This possible involvement of other proinflammatory cytokines could explain why IFN-γ knock-out mice were afforded only partial protection when used in our anti-CD40 toxicity model. In nonirradiated mice, IL-12 administered in combination with either IL-2 [21] or IL-18 [24] has been shown to elicit rapid mortality within 4 to 6 days that is characterized by destruction of normal gut architecture. Given the similarities in the rate of morbidity and the intestinal lesions following administration of IL-12 to those observed following administration of anti-CD40 MoAb, we evaluated for the presence of IL-12 in mice receiving anti-CD40 MoAb after lethal TBI toxicity. We observed marked increases of IL-12 p40 in the serum of mice receiving anti-CD40 MoAb compared to that of control mice, suggesting a role for IL-12 p40 in anti-CD40–mediated toxicity. We next evaluated the role of IL-12 in the CD40 toxicity model using IL-12 p40 knock-out mice. These data demonstrated that an absence of IL-12 p40 completely eliminated anti-CD40 MoAb–induced mortality and pathology, further supporting the role of IL-12 as a mediator of toxicity following lethal TBI and CD40 stimulation. These data, combined with our previous data showing an increase of IFN-γ following lethal TBI and CD40 stimulation, support the role of

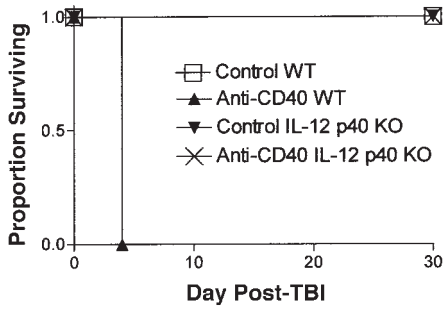

Figure 8. Absence of IL-12 p40 improves survival in anti-CD40 MoAb–treated mice. BALB/c WT and IL-12 p40 knock-out (KO) mice received 750 cGy TBI and a syngeneic BMT, followed by daily IP injections of control serum or 10 μg of anti-CD40 MoAb for 4 days. Mice deficient in IL-12 p40 receiving anti-CD40 MoAb demonstrated significantly ($P = .0009$) increased survival compared to similarly treated WT mice. Experiments were performed twice using 7 to 10 mice.

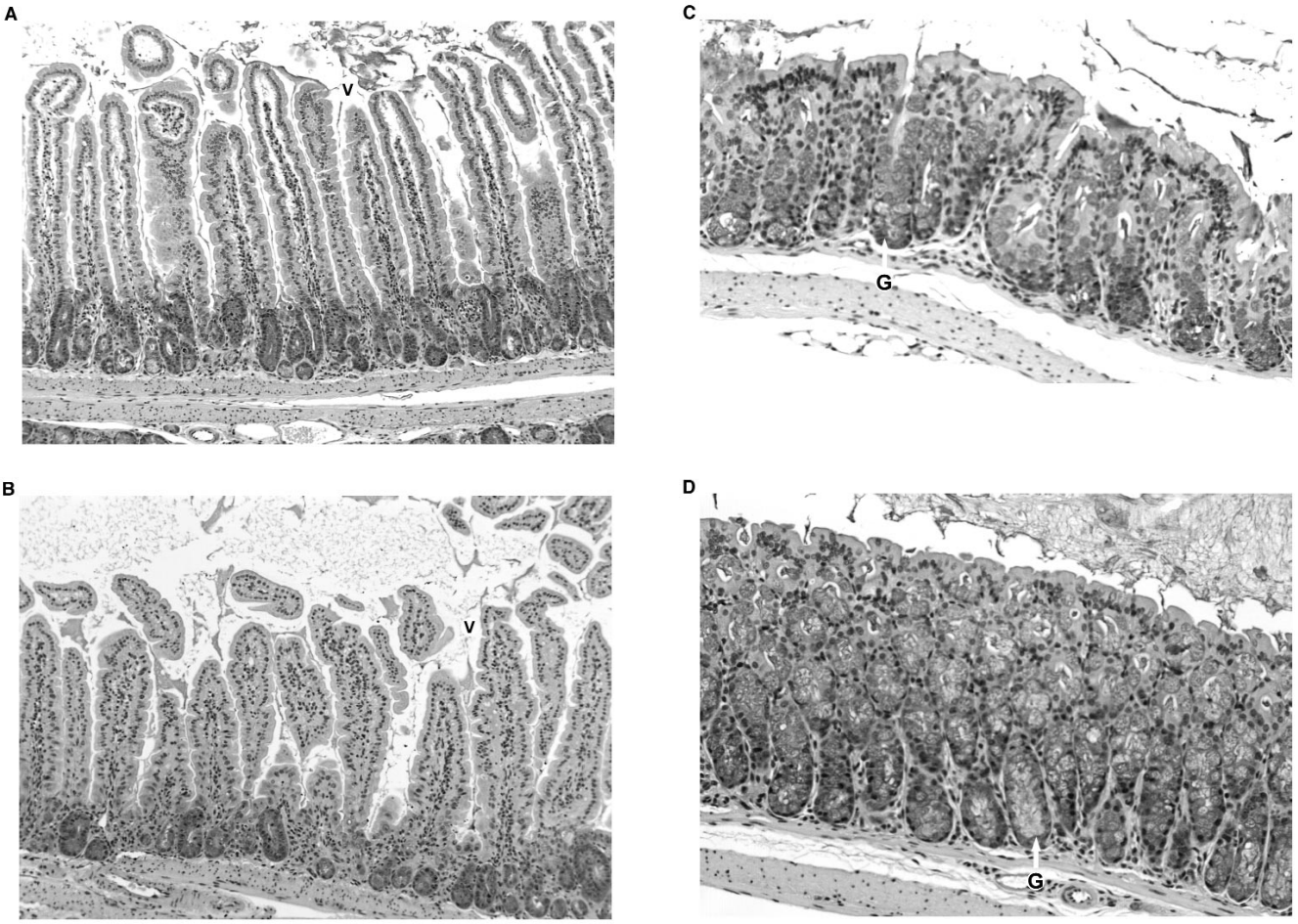

Figure 9. Elimination of IL-12 p40 protects small intestine and colon from anti-CD40 MoAb–mediated destruction. A and C, BALB/c IL-12 p40 knock-out mice treated with control serum. Small intestine (9A) has normal-length villi (V), and the colon (9C) has crypts with normal populations of goblet cells (G). B and D, BALB/c IL-12 p40 knock-out mice treated with anti-CD40 MoAb. Small intestine (9B) has normal-length villi (V), and the colon (9C) has crypts with normal populations of goblet cells (G), compared to similarly treated WT mice (Figure 2 and 3). Experiments were performed 2 times with 1 to 3 mice undergoing histological evaluation in each experiment. Hematoxylin and eosin, original magnification ×10.

IFN-γ as an enhancer of IL-12 secretion from macrophages [23] and suggest that these two cytokines may act together as mediators of pathogenesis during an inflammatory process via a positive feedback mechanism between IL-12–producing macrophages and IFN-γ–producing T- and NK cells.

One possible scenario involving both IL-12 and IFN-γ as mediators of toxicity following anti-CD40 administration could occur via the enhancement of CD40 expression on macrophages by IFN-γ [25], as well as the dependence of macrophages on CD40-CD40L interactions [26,27] and IFN-γ [23] for the production of IL-12. Given the role of CD40 in the production of IL-12 from macrophages and the implication of macrophages as mediators of IL-12 toxicity [21,24], it is possible that CD40 stimulation via an agonistic MoAb and the resulting increase in the levels of IFN-γ [13] trigger the production of large amounts of IL-12. Because IL-12 enhances the secretion of IFN-γ from T- and NK cells [23], a mechanism of uncontrolled cytokine production could be established in which the positive feedback amplification of IL-12 production is enhanced by the production of IL-12–induced IFN-γ. It is also possible that CD40 acts early in this pathway, with CD40 stimulation of macrophages inducing the secretion of multiple proinflammatory cytokines including IL-1, IL-6, and TNF, whereas the addition of rmIL-12 leads primarily to the production of IFN-γ. This theory would explain why the absence of IFN-γ affords complete protection from rmIL-12–mediated toxicity, whereas anti-CD40–treated IFN-γ–deficient mice are only partially protected. It would be of interest to evaluate whether IFN-γ is the sole mediator of IL-12–induced toxicity in the CD40 model, and experiments using IL-12 p40 knock-out and IFN-γ knock-out mice in the same experiment are currently being performed. In addition, it would be of interest to neutralize IL-12 in IFN-γ knock-out mice.

Clearly conditioning plays a role in the observed toxicity because unirradiated mice experience no toxic effects when given up to 100 μg of anti-CD40 MoAb or 1.0 μg rmIL-12. Intense myeloablative conditioning, either by irradiation or chemotherapy, triggers an inflammatory cytokine cascade resulting in the damage of intestinal mucosa and the secretion of proinflammatory cytokines such as IL-1, IL-6, and TNF-α. It is of interest to note that whereas lethally irradiated BALB/c and CB.17/SCID mice were susceptible to

both anti-CD40 MoAb– and rmIL-12–induced toxicity, similarly irradiated C57BL/6 mice required 10 times the amount of anti-CD40 MoAb to show the same toxic effects. In addition, lethally irradiated C57BL/6 mice were completely resistant to up to 1.0 μg of rmIL-12. These data suggest strain differences and support previous work by Sykes et al. [22], who demonstrated no toxic effects following the administration of rmIL-12 to lethally irradiated C57BL/6 mice that received a single fraction of lethal irradiation on the day of bone marrow transplantation. In addition, Sykes et al. [22] demonstrated that IFN-γ–deficient mice were resistant to rmIL-12–induced toxicity, further supporting our data obtained using IFN-γ knock-out mice.

The question of whether destruction of the gut by anti-CD40 MoAb and rmIL-12 occurs directly or indirectly remains unanswered. Gut lesions observed in our IL-12 and CD40 toxicity models could be the result of direct targeting of the normal gut tissue by IL-12 and an anti-CD40 MoAb, and we are currently evaluating the direct effects of CD40 using CD40 knock-out mice. Conversely, toxicity could be occurring via the activation of other effector cell populations, such as macrophages or NK cells, which could modulate tissue destruction via the production of inflammatory cytokines. Although others have proposed a role for NK cells in gut pathogenesis [24], we found that the depletion of NK cells had no effect on either rmIL-12– or anti-CD40 MoAb–mediated toxicity (data not shown).

In conclusion, the data presented here demonstrate that the administration of either anti-CD40 MoAb or rmIL-12 to lethally irradiated mice induces acute morbidity in lethally irradiated mice, and the toxicity is mediated, at least in part, by the production of IFN-γ. Anti-CD40 MoAb has demonstrated potent antitumor effects when used in combination with IL-2 against murine solid-tissue carcinomas (unpublished data). In addition, clinical trials evaluating IL-12 in combination with IL-2 as an immunotherapy for cancer are currently in progress. Given the toxicity of high doses of inflammatory cytokines, it is hoped that lower doses could be used to treat minimal residual disease following the reduction of tumor burden in the host with either chemotherapy or irradiation. However, synergy between IL-2 and IL-12 in the induction of toxicity has been demonstrated using lethally irradiated mice [28]. This finding, as well as those of our studies, suggests that anti-CD40 and rmIL-12 must be used prudently in patients receiving myeloablative therapy.

ACKNOWLEDGMENTS

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract no. N01-CO-124000 and supported in part by National Institutes of Health Grants RO1 AI34495, RO1 HL63452, and R37 HL56067.

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