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ORIGINAL ARTICLE

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Therapeutic effect of colon tumor cells expressing FLT-3 ligand plus systemic IL-2 in mice with syngeneic colon cancer

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Abstract Flt-3 ligand (FL) and interleukin-2 (IL-2) have been shown to enhance individually the antitumor response against several cancers. Therefore, treatment with a combination of FL gene-transduced tumor cell plus soluble IL-2 was studied in a murine colon adenocarcinoma model. The human full-length FL cDNA was cloned from FL-expressing cell line AML-193 by reverse transcription–polymerase chain reaction (RT–PCR). CC-36 colon tumor cells were transduced with the FL gene (CC-36-FL). In vivo and in vitro secretion of FL from CC-36-FL was confirmed by enzyme-linked immunosorbent assay (ELISA). Moreover, enhancement of dendritic cells in vivo was evaluated in mice transplanted with CC-36-FL. The therapeutic efficacy of CC-36-FL plus systemic IL-2 was tested using six groups ($n = 12$–13/group) of 10-week-old male Balb/c mice transplanted with 10^3 CC-36 tumor cells. Mice were treated subcutaneously with 10^6 irradiated CC-36 cells, 10^6 irradiated CC-36 cells + IL-2, 10^6 irradiated CC-36-FL cells, 10^6 irradiated CC-36-FL + IL-2, or IL-2 alone on days 4, 10 and 18 after tumor transplantation. A group of mice with no treatment served as a control. All of the treatment injections were performed subcutaneously in the left flank. IL-2 (2×50,000 IU) was administered intraperitoneally in 3-day cycles (days 4–6, 10–12, 17–19). Tumor growth was determined by measuring the tumor diameter. A survival experiment was performed with the same treatments, and mice were observed for survival for 100 days. The group of mice treated with the combination of CC-36-FL + IL-2 showed a significant reduction in tumor burden when compared to the no treatment group and the other control treatment groups ($P < 0.05$). Similarly, the group of mice treated with CC-36-FL + IL-2 displayed significant survival when compared with the other control groups ($P < 0.05$). In Balb/c mice, the CC-36-FL plus systemic IL-2 therapy significantly decreased the tumor burden and increased the survival rate when compared to mice treated by control therapies or mice that received no treatment.

Keywords CC-36-FL + systemic IL-2 therapy · Colon tumor cell · Survival

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Introduction

The cytokine Flt-3 ligand (FL) is a naturally occurring glycoprotein that is expressed as a membrane-bound protein that undergoes proteolytic cleavage to form a soluble protein [12, 16]. Both the membrane-bound and the soluble protein forms are biologically active [15]. FL, particularly in combination with other cytokines, augments the expansion of hematopoietic progenitor cells (HPC) and the development of monocytes, neutrophils, natural killer (NK) cells, B cells, and T cells [15]. A unique capacity of FL, more importantly, is the profound expansion of functionally active dendritic cells (DC) in multiple lymphoid [18, 28, 29] and non-lymphoid tissues [25] in mice. Likewise, in vitro and ex vivo studies have shown that FL directly stimulates proliferation of $CD34^+$ stem cells into DC progenitors [20, 23].

Given the well-documented central role of DC in primary and secondary immune responses [1], many investigators have tested whether FL-induced DC confers enhanced antitumor activity. Indeed, FL has been shown to retard tumor growth and stimulate tumor-specific immune responses in several murine tumor models [2, 3, 4, 5, 6, 9, 11, 17, 21, 32]. Recent studies have also shown that FL-induced NK activity may be another source of antitumor immunity [3, 7, 11, 21, 25].

It has been well established that the systemic administration of IL-2 or the transplantation of IL-2-producing cells or vectors exerts a wide range of immunologic effects in vivo, including the proliferation of T cells, NK cells and lymphokine-activated killer cells [10, 22, 30]. Moreover, systemic administration of interleukin 2 (IL-2) in DC-based immunotherapy has been shown to increase the induction of an antitumor effect [13]. Therefore, a combination therapy of FL with IL-2 could synergistically produce improved antitumor activity through expanding DC by FL for an increased stimulation of antigen-specific T cells, and expanding the stimulated antigen-specific T cells by IL-2. This hypothesis was tested in the present study using FL-gene transduced colon carcinoma cells and soluble IL-2 in a CC-36 colon adenocarcinoma model. Moreover, this study is the first to detail a therapy involving a FL-transduced autologous tumor cell line in combination with systemic IL-2 in a murine syngeneic colon cancer model [14, 19].

Materials and methods

Mice

Normal and athymic male BALB/c mice, 8 and 6 weeks of age, respectively, were obtained from Harlan-Sprague-Dawley (Indianapolis, Ind.) and allowed to become acclimatized for 2 weeks prior to use. Animals were housed in the Saint Vincent's Hospital and Medical Center of the New York Surgical Research Animal Facility under pathogen-free conditions, controlled temperature and humidity, and a 12-h light/dark cycle at a temperature of 20–22°C with food and water available ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee.

Cell lines

The CC-36 cell line is a murine well-differentiated colorectal adenocarcinoma induced with 1,2-dimethylhydrazine dihydrochloride [8]. The ascetic CC-36 colon adenocarcinoma cell line was kindly provided by T.H. Corbett and D.P. Griswold (Southern Research Institute, Birmingham, Ala.). This ascetic CC-36 tumor cell line was adapted in tissue culture by Sato et al. [24] in our laboratory. The CC-36 cell line was maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM/ml pyruvate, 1% non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% multivitamins at 37°C in 5% CO_2. Similarly, FL gene-transduced CC-36 tumor cell lines were maintained in the same medium. A single-cell suspension of either parental or FL-transduced tumor cell line was prepared by stripping the cell monolayer with trypsin–ethylene diamine tetra-acetic acid (EDTA), washing the cells once with phosphate-buffered saline (PBS), and resuspending the cells in Hank's balanced salt solution (HBSS) at a concentration of 10^3 or 10^4 viable cells/100 μl (all media, supplements and reagents were obtained from Gibco BRL, Grand Island, N.Y.).

FL gene-transduced CC-36 cells

The human FL cDNA was cloned from a FL-expressing cell line, AML-193 (American Type Culture Collection, Manassas, Va.) using reverse transcription–polymerase chain reaction (RT–PCR). In brief, mRNA was isolated from AML-193 using a commercially available standard mRNA isolation kit (Sigma, Saint Louis, Mo.). The mRNA was converted into cDNA using a first strand cDNA synthesis kit from Boehringer Mannheim (Indianapolis, Ind.). This cDNA was used as a template for amplifying FL-specific cDNA with appropriate sense and non-sense primers using a PCR kit (Boehringer Mannheim, Indianapolis, Ind.). The amplified FL-cDNA was confirmed for specific molecular size and then cloned into mammalian expression vector pCR 3.1 using a TA-cloning kit (Invitrogen, San Diego, Calif.) and transferred to DH5α competent cells (Gibco BRL, Grand Island, N.Y.). The transformed DH5α bacteria were selected on LB-amp agar plates. The pCR 3.1 plasmid encoding the FL cDNA (pCR 3.1-FL) was isolated and sequenced using an automated sequencer (ABI Prism, model 377) using appropriate sense and non-sense primers. The FL cDNA sequences from several plasmids were compared with the published FL cDNA sequence from the National Gene Database. The orientation of the FL cDNA sequence in the pCR 3.1-FL was determined using various restriction enzymes. Colony No. 17 contained pCR 3.1-FL with the correct FL cDNA orientation. This colony was expanded for the preparation of pCR 3.1-FL plasmid DNA. The pCR 3.1-FL plasmid DNA was purified using a commercially available plasmid DNA isolation kit (Qiagen, Santa Clarita, Calif.).

Transduction of pCR 3.1-FL DNA into CC-36 colon tumor cells was performed using the Lipofectamine reagent (Gibco BRL, Grand Island, N.Y.) according to the manufacturer's instructions. Briefly, $1–2\times10^5$ CC-36 cells were cultured in a 35 mm dish for 24 h in a CO_2 incubator. Cells were washed once with serum-free medium to which 0.8 ml of transfection buffer containing 1–2 μg of pCR 3.1-FL cDNA was added. After 6 h incubation at 37°C, 1 ml of fresh medium was added and incubated for 24 h in a CO_2 incubator. The medium was replaced with selection medium containing G418 (400 μg/ml) (Sigma, St. Louis, Mo.) and cultured for 1 week. Cells were stripped and cloned in a 96-well plate by limiting dilution method with one or less than one cell per well. Several CC-36-FL clones were developed and expanded. Culture supernatants from these clones were harvested and tested for FL using a commercially-available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, Minn.) and by immuno dot-blot using specific antibody to FL (kindly provided by Immunex, Seattle, Wash.). Eleven FL-positive clones were selected and further characterized for the production of membrane and soluble FL.

Characterization of CC-36-FL

The aforementioned transduced CC-36-FL clones and parental CC-36 cell line were characterized for the expression of soluble and membrane FL using an ELISA kit and immunofluorimetry, respectively. Supernatants were collected from 10^6 cells kept in 5 ml of medium for 24 h in a CO_2 incubator. These supernatants were used in an ELISA kit according to the manufacturer's instructions to measure soluble FL. Results are represented as ng/10^6/24 h per clone. Moreover, expression of membrane-bound FL was evaluated with a FITC-conjugated monoclonal antibody (PharMingen, San Diego, Calif.) specific to a recombinant human FL, and analyzed by FACScan (Becton Dickinson, San Jose, Calif.). Appropriate isotype-matched monoclonal antibodies (PharMingen, San Diego, Calif.) were included as immunofluorimetric controls.

To evaluate the bioavailability of FL from CC-36-FL, mice were injected with 10^6 irradiated (3,000 rads) CC-36-FL tumor cells subcutaneously in the right flank in a total volume of 100 μl HBSS/injection. Irradiated CC-36-FL cells were used in the experiments, as previously determined. Plasma samples were collected by killing the mice on 9 consecutive days, including a zero time-point (30 min after irr-CC-36-FL injection). At least three mice were used for each time-point. Plasma samples from three non-treated mice served as a negative control. Levels of FL in the plasma samples were determined using a commercially-available ELISA kit according to the manufacturer's instructions (R&D Systems, Minn.).

Measurement of circulating DC

To determine the role of CC-36-FL in the enhancement of mobilized, circulating DC in mice transplanted with CC-36-FL, the animals were injected with 1 million irradiated (10,000 rads) CC36-FL tumor cells subcutaneously. The mice were killed at several time points (days 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10). At least three mice were used per time point. Blood samples from three mice were pooled and peripheral blood mononuclear cells (PBMC) were isolated. The PBMC were stained indirectly with mouse DC-specific antibody, DEC-205 (Serotec, U.K.). The number of cells positive to DEC-205 per million PBMC was measured using FACScalibur (Becton Dickinson, Calif.). At least 50,000 events were set to capture the low-percentage DC in the FACS analysis. An isotype-matched control (Serotec, U.K.) was also used in the FACS analysis. Bone marrow-derived 7-day cultured (in murine GM CSF + IL-4) DC were used as a positive DC control in this analysis.

In vivo growth characteristics of CC-36-FL

To evaluate potential in vivo autocrine FL-induced reduction or enhancement of CC-36 tumor growth, athymic Balb/c mice ($n = 5$/group) were transplanted with 10^3 parental CC-36 or CC-36-FL tumor cells subcutaneously in the right flank in a total volume of 100 µl of HBSS. Tumor incidence (i.e., ≥3 mm mean tumor diameter) was recorded in both groups every 4–5 days post-tumor inoculation.

Antitumor efficacy of CC-36-FL + systemic IL-2

To assess the antitumor efficacy of CC-36-FL + systemic IL-2 therapy, the following experimental protocol was performed. Immunocompetent Balb/c mice were transplanted with 10^3 parental CC-36 colon tumor cells subcutaneously in the right flank. Random designations of treatment and control groups ($n = 12$–13/group) were as follows: (1) 10^6 irradiated CC-36 cells (alone or in combination with IL-2); (2) 10^6 irradiated CC-36-FL cells (alone or in combination with IL-2); (3) IL-2 alone; or (4) a no-treatment control. All irradiated (10,000 rads) CC-36-FL or irradiated CC-36 tumor cell immunizations were administered subcutaneously in the opposite left flank on days 4, 11 and 18 after parental CC-36 tumor cell transplantation. Injected CC-36/CC-36-FL cells were >90% viable as determined by trypan blue exclusion. IL-2 (50,000 IU×2 injections 12 h apart) was administered intraperitoneally over 3-day cycles (days 4–6, 11–13 and 18–21 post-tumor challenge) in a total volume of 50 µl PBS per injection. Tumor growth was monitored every 2–3 days. All palpable tumors were measured in two dimensions using vernier calipers and recorded as mean tumor diameter.

A survival experiment was performed with the above groups and the same treatments. Survival was observed on a daily basis for a total of 100 days following tumor inoculation. Death due to tumor burden was confirmed by autopsy. The above experiments were repeated once independently.

Cytolytic assay

CC-36 lytic activity of peripheral blood lymphocytes (PBL) from the group of mice treated with CC-36-FL + systemic IL-2 was measured using standard chromium release methodology. PBL from mice transplanted with CC-36 served as a control. Freshly prepared lymphocytes or lymphocytes co-cultured with CC-36 lysate-pulsed DC for 5 days in RPMI medium containing 10 units of interleukin-2 were tested in the assay. For the assay, target cells CC-36 or Yac-1 were harvested, washed, and incubated with 100 µCi sodium chromate (^{51}Cr; Amersham)/10^6 cells, in 0.5 ml volume for 60 min at 37°C. After washing the cells in RPMI 1640 four times, radiolabeled target cells were added to U-bottomed microtiter plates (Costar) at a concentration of 10^4 cells in 100 µl of medium. Effector (E) lymphocytes to target (T) tumor cell ratios ranged from E:T: 80:1–10:1. At least four replicates were used per ratio. Plates were spun at 250 g for 5 min and were incubated at 37°C in a humidified incubator for 4 h. To determine maximum release, 0.1 ml of 1% sodium dodecyl sulfate (SDS) solution was added to the appropriate wells. At the end of 4 h incubation, the supernatants from the wells were harvested on fiber filters utilizing a Skatron Collection System and then counted for 1 min using a gamma counter. Counts per minute (cpm) was used to calculate percent release (%R) according to the following formula:

$$\%R = \frac{Experimental\ release - spontaneous\ release}{Maximum\ release - spontaneous\ release} X100$$

FL-induced interleukin-12 and interferon-gamma production

Since FL can induce the production of interleukin-12 (IL-12) and interferon-gamma (IFN-γ), IL-12 and IFN-γ levels were examined in mice transplanted with CC-36-FL. For this experiment, normal Balb/c mice were injected s.c. with 1 million irradiated (10,000 rads) CC36-FL tumor cells. The mice were killed at several time points (days 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10), and plasma samples were collected. At least three mice were used for each time point. Murine IL-12 and IFN-γ levels were measured in the plasma samples using a commercial ELISA kit according to the manufacturer's instructions (R&D Systems, Minn.).

Statistical analysis

Tumor measurements are presented as means tumor diameter ± SEM. Comparisons between the experimental groups were performed using the 2-tailed Student's t-test. Differences were considered significant when $P < 0.05$. Wilcoxon statistics were used to analyze survival of mice in the survival study.

Results

Characterization of the CC-36-FL cell line

Given the fact that both forms of the FL protein (i.e. soluble and membrane-bound) have clearly demonstrated biological activity [15], full-length FL cDNA was cloned. The DNA sequence of the FL cDNA was found to be same as that of the published FL cDNA sequence, except for an alteration in the downstream position at 683 where nucleotide C was replaced with T. However, this alteration produced a codon for the same amino acid. Using this FL cDNA, several FL gene-transduced CC-36 colon tumor cell clones were developed and tested for the in vitro expression of soluble FL. Clones 2, 8, and 10 were found to express the highest levels of soluble FL in their supernatants, which ranged from 40 to 80 ng per million cells in 24 h. Clone 2 expressed the highest level of FL; i.e., 80 ng/10^6 cells in 24 h. In addition, continuous FL secretion was observed in the latter clone for over 1 year, which suggests its long-term stability in producing FL. Therefore, this CC-36-FL clone was selected for further characterization and therapeutic studies. Moreover, it is interesting to note that the amount of FL secreted by this clone is comparable to that of other previously reported FL-transduced

tumor cells [9, 18]. As expected, the parental non-transduced CC-36 colon tumor cells did not express any detectable levels of soluble FL.

Subsequently, the CC-36-FL clone was tested for the presence of membrane-bound FL. As can be seen in the FACS analysis (Fig. 1), this clone expressed membrane-bound FL. The parental CC-36 cells, however, did not show any detectable membrane-bound FL.

Furthermore, soluble FL in the plasma of mice transplanted with the CC-36- FL clone was studied to determine the bioavailability of FL. FL could be detected in plasma at a concentration of 300 pg/ml as early as 24 h post-transplantation (Fig. 2). Moreover, FL was present at detectable levels until day 9 post-CC-36-FL transplantation. However, plasma FL concentrations declined sharply after 24 h and did not return to similar concentrations during the 9 days follow-up. This could be due to the utilization of FL after the initial 24 h, or to a reduction in the secretion of FL from CC-36-FL after the 24-h time point. Plasma samples from normal mice did not contain any detectable levels of FL.

The tumor-growth-promoting or -retarding effect of FL on CC-36-FL was determined in T-cell deficient Balb/c nude mice. The latter were used in this study to eliminate any tumor-retarding effect due to FL-induced antitumor T-cell response. The tumor incidence amounted to 100% for both CC-36 FL and parental CC-36 transplanted groups during the 2-month follow-up period (Fig. 3). This experiment shows that the FL from CC-36-FL had no autocrine effect on tumor incidence.

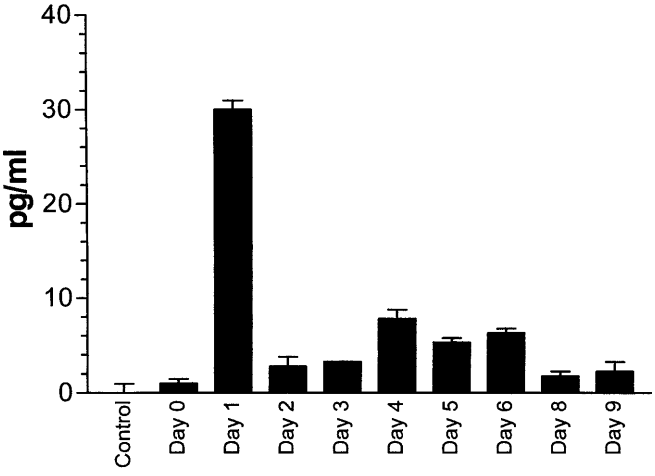

Fig. 2 Circulating plasma levels of FL were determined in mice ($n=3$) transplanted with 10^6 parental CC-36-FL at various time intervals (days 1–9). The plasma level of FL peaked 24 h after CC-36-FL transplantation. Plasma from normal mice ($n=3$) showed no FL. The FL could be detected in plasma at 9 days post-CC-36-FL transplantation

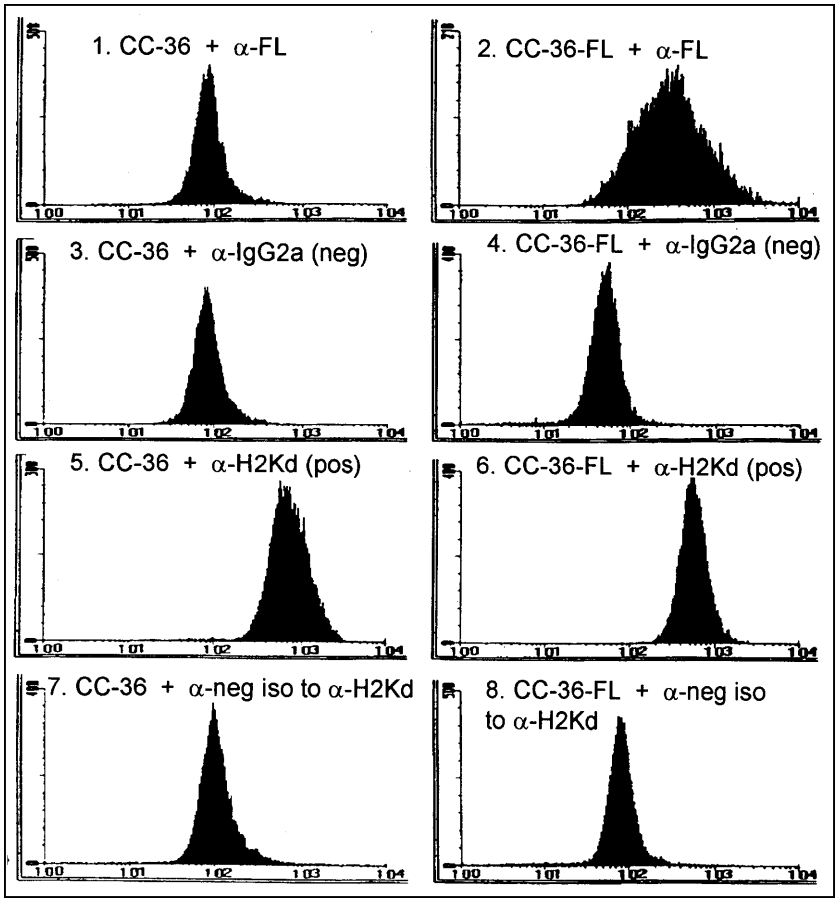

Fig. 1 Clone 2 was analyzed for the membrane expression of FLT3-L by FACS analysis. *Plate 2* shows the expression of membrane FLT3-L on CC-36-FL clone 2, whereas the parental cells (*plate 1*) did not show any FLT3L expression. The isotype-matched controls on CC-36 and CC-36 FL (*plates 3 and 4*) did not have any fluorescence. The expression of MHC class II antigen H2Kd was observed in both CC-36 and CC-36-FL

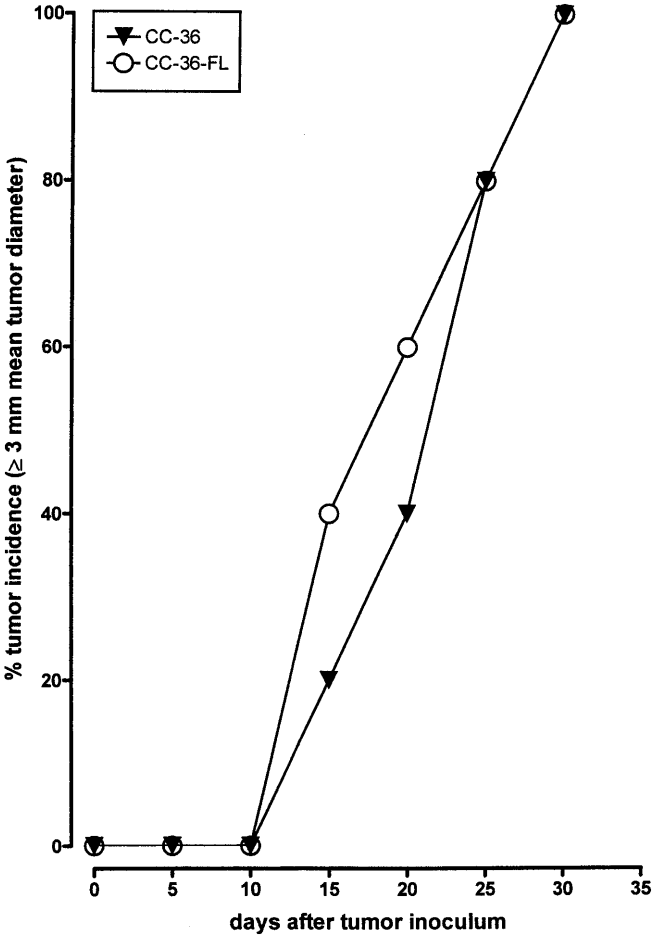

Fig. 3 Tumor incidence of CC-36 (-O-) ($n = 5$) and CC-36-FL (-▼-) ($n = 5$) in Balb/c nude mice. Mice were transplanted with 10^4 parental CC-36 or CC-36-FL tumor cells injected s.c. in the right flank in a total volume of 100 µl Hank's balanced salt solution (HBSS)/injection. Tumor incidence (≥3 mm mean tumor diameter) was recorded over 30 days post-tumor transplantation

Mobilized circulating DC in mice transplanted with CC-36-FL

After establishing the in vivo and in vitro expression of FL by CC-36-FL cells, the effect of CC-36-FL in augmenting the circulating DC was studied in Balb/c mice transplanted with CC-36-FL. The kinetics of circulating DC has been presented in Fig. 4. PBMC from mice transplanted with CC-36-FL contained 6,000 DC per million PBMC at day 2 post-tumor transplantation, and reached a maximum of 190,000 DC per million PBMC at day 5. There was a sharp decline in the number of DC in the PBMC at day 6, followed by a gradual increase after day 6, which reached a peak of 250,000 DC per million PBMC on day 9. After that point, DC were maintained at levels above 3,500 per million PBMC. The normal Balb/c mice displayed a range of 1,700–3,500 DC per million PBMC. Therefore, when compared with normal mice, mice transplanted with CC-36-FL showed an exponential increase in circulating DC (Fig. 4). Although these results clearly show an

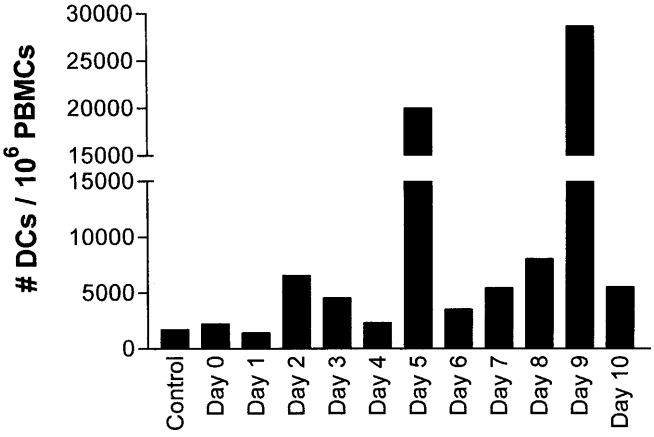

Fig. 4 CC-36-FL induced DC response was measured in mice transplanted with 1 million irradiated CC-36-FL s.c. Mice were killed at several time points (days 0–10). PBMC were isolated from pooled blood from three mice at each time point and stained with DC-specific monoclonal antibody DEC-205. The number of cells positive to DEC-205 was measured using FACS analysis. Results are expressed as number of DC per million PBMC. Mice transplanted with CC-36-FL showed an increased number of DC in PBMC when compared with PBMC from normal mice. The DC response was noted as early as 48 h after CC-36-FL transplantation and continued for at least 10 days

increase in circulating DC in mice transplanted with CC-36-FL, it is not known why DC peak at days 5 and 9. It is possible that DC levels are increased in PBMC with the production of FL from CC-36-FL, and that the number of these accumulated DC reaches a maximum on day 5. Later, the DC mature and are subsequently cleared by apoptosis. This cycle may be repeated on day 9. It is also interesting to note that there is a correlation between the enhancement of circulating DC and the increased levels of FL in the plasma (Fig. 2).

CC-36-FL + IL-2 antitumor therapy in mice bearing syngeneic CC-36 colon cancer

Having established FL-expression both in vivo and in vitro by the CC-36-FL cell line and the enhancement of circulating DC in mice transplanted with this cell line, the antitumor efficacy of CC-36-FL + IL-2 combined therapy was tested in mice transplanted with parental CC-36 tumor cells. When compared with the no treatment, IL-2 alone, CC-36 alone, CC-36 + IL-2, and CC-36-FL alone control groups, the CC-36-FL + IL-2 therapy group displayed significantly lower tumor growth kinetics (Fig. 5; $P < 0.05$). At the 6-week interval, the mean tumor diameters (± SE) of the control, IL-2 alone, CC-36 alone, CC-36-FL alone, CC-36 + IL-2 and CC-36-FL + IL-2 groups were 8.6 ± 3.3, 5.6 ± 2.4, 1.8 ± 0.8, 5.3 ± 1.9, 7.1 ± 2.3 and 0.8 ± 0.5 mm, respectively. At the 9-week interval, a more prominent antitumor effect was observed with this therapy. The mean tumor diameters (± SE) of the control, IL-2 alone, CC-36 alone, CC-36-FL alone, CC-36 + IL-2 and CC-36-FL + IL-2 groups were 25.3 ± 5.2, 17.2 ± 4.2,

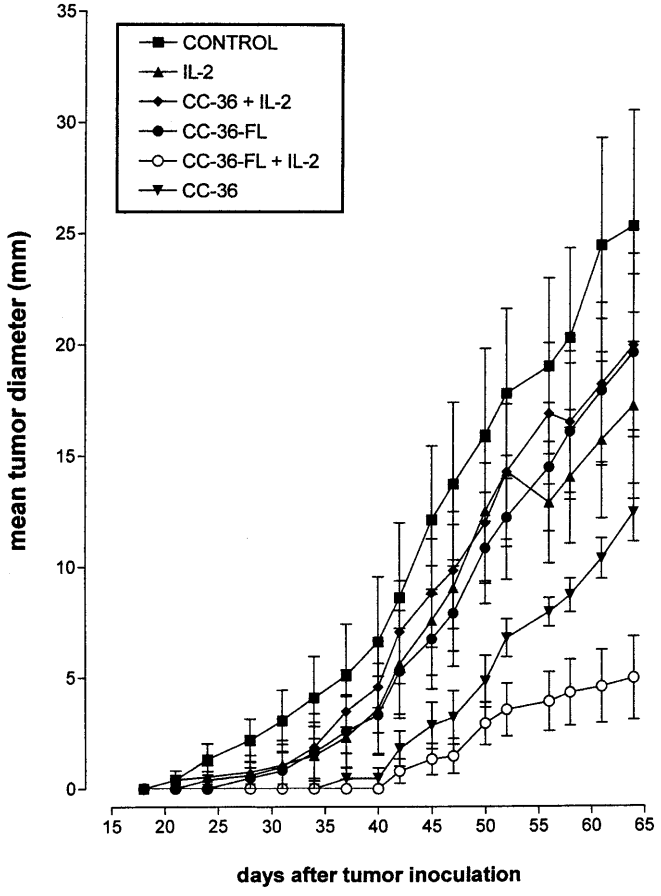

Fig. 5 Antitumor effect of CC-36-FL + IL-2 combined immunotherapy in mice transplanted with parental CC-36 tumor cells. Mice were transplanted with parental CC-36 colon tumor cells 10^3 s.c. in the right flank. Mice were treated with 10^6 irr-CC-36 cells (-▼-), 10^6 irr-CC-36 cells and IL-2 (-◆-), 10^6 irr-CC-36-FL cells (-●-), 10^6 irr-CC-36-FL cells plus IL-2 (-O-), or IL-2 alone (-▲-). All irradiated (10 Gy) CC-36-FL or CC-36 tumor cell immunizations were administered s.c. in the opposite left flank on days 4, 11 and 18 after tumor transplantation. IL-2 (50,000 IU×2 injections, 12 h apart) was administered intraperitoneally over 3-day cycles (days 4–6, 11–13 and 18–21 post-tumor challenge) in a total volume of 50 µl PBS/injection. Tumor growth was monitored every 2–3 days. A treatment-free control was also used in this experiment (-■-). The CC-36-FL + IL-2 combined therapy when compared with the treatment-free control, IL-2 alone, CC-36 alone, CC-36 + IL-2 and CC-36-FL alone controls produced significantly ($P < 0.05$) less tumor growth kinetics

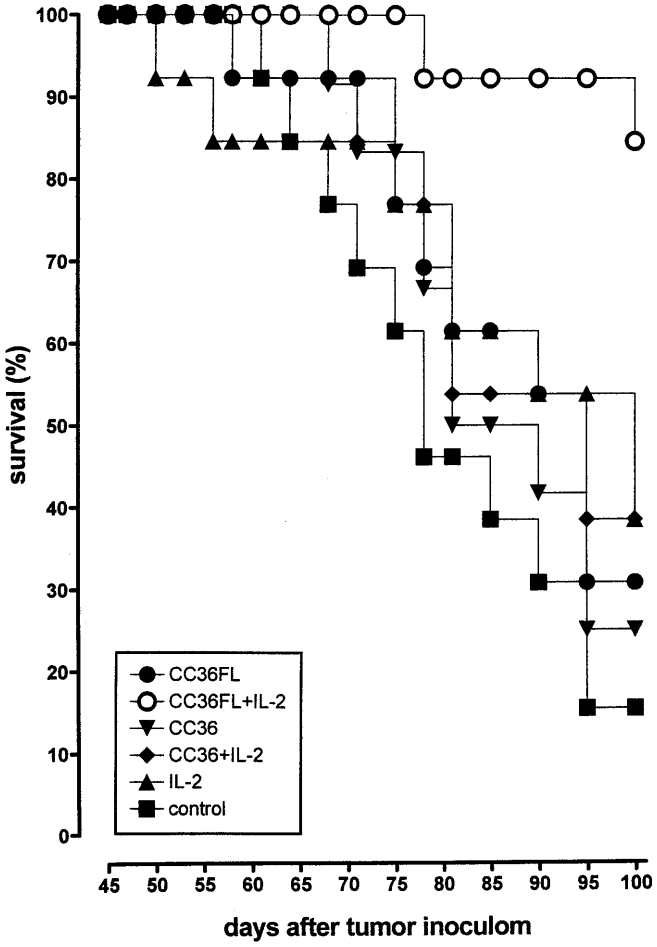

Fig. 6 A survival study was performed to determine efficacy of CC-36-FL + IL-2 combined immunotherapy in mice transplanted with parental CC-36 tumor. Mice were transplanted with parental CC-36 colon tumor cells 10^3 s.c. in the right flank. Mice were treated with 10^6 irr-CC-36 cells (-▼-), 10^6 irr-CC-36 cells and IL-2 (-◆-), 10^6 irr-CC-36-FL cells (-●-), 10^6 irr-CC-36-FL cells plus IL-2 (-O-), or IL-2 alone (-▲-). All irradiated (10 Gy) CC-36-FL or CC-36 tumor cell immunizations were administered s.c. in the opposite left flank on days 4, 11 and 18 post-tumor transplantation. IL-2 (50,000 IU×2 injections, 12 h apart) was administered intraperitoneally over 3-day cycles (days 4–6, 11–13 and 18–21 post-tumor challenge) in a total volume of 50 µl PBS/injection. A treatment-free control was also used in this experiment (-■-). Mice were followed for survival for 100 days after tumor transplantation. The CC-36-FL + IL-2 combined therapy when compared with the treatment-free, IL-2 alone, CC-36 alone, CC-36 + IL-2 and CC-36-FL alone control groups showed a significant survival rate ($P < 0.05$)

12.5 ± 1.3, 19.9 ± 3.5, 20.0 ± 4.1 and 4.9 ± 1.8 mm, respectively. When compared to the other groups, the group of mice treated with CC-36-FL + IL-2 showed significantly less tumor burden ($P < 0.01$). Although the group treated with CC-36 alone initially displayed a lower tumor burden (at 4- and 6-week intervals), later (at the 9-week interval), a significantly higher tumor burden was observed in this group than in the group treated with CC-36-FL + IL-2. The IL-2 alone or FL-producing CC-36 alone groups did not produce a significant reduction in tumor burden when compared to the CC-36-FL + IL-2 group. This clearly shows that both CC-36-FL and IL-2 are necessary to induce the observed anti-tumor response. Furthermore, 50% (5/10) of the CC-36FL + IL-2 combined therapy-treated mice were tumor-free for over 2 months following tumor transplantation.

In addition, in a survival study the above-mentioned combination therapy produced an antitumor effect that was proportional to tumor growth kinetics (Fig. 6). The mean survival (± SE) of the no treatment control, IL-2 alone, CC-36 alone, CC-36 + IL-2, CC-36-FL alone control, and the CC-36-FL + IL-2 combined therapy groups were 51 ± 9, 59 ± 9, 64 ± 10, 64 ± 10, 61 ± 8 and

76 ± 7 days, respectively at 100-days follow-up. In addition, it is interesting to note that over 80% of mice in the combined therapy group survived for 100 days following tumor challenge.

Cytotoxicity against CC-36

In order to understand the mechanisms involved in the induction of cytotoxic T cell response by CC-36 + IL-2 therapy, fresh PBL from mice treated with the latter therapy were tested in a cytotoxicity assay using parental CC-36 cells and NK-sensitive Yac-1 cells as targets. Fresh PBL did not show any CC-36 lytic activity (data not shown). However, when the PBL were stimulated with syngeneic DC pulsed with CC-36 lysate and cultured for 5 days in IL-2 medium, this lysed both CC-36 and Yac-1 cells (Fig. 7). The CC-36 lytic activity of PBL from the treatment group at an E:T of 80:1 was higher than that of PBL from the no-treatment control group. Moreover, PBL from the treated mice showed a similar percentage lysis against both CC-36 and Yac-1 targets. Therefore, the observed CC-36 lytic activity of PBL from the treatment group may not be specific to CC-36. NK and LAK activity could have contributed to this CC-36 lytic activity, since the mice were treated with IL-2. Moreover, FL from CC-36-FL could have contributed to the augmentation of NK and LAK activity.

IL-12 and IFN-γ in CC-36-FL-treated mice

Since FL can stimulate the production of IL-12 from monocytes, IL-12 levels were determined in the plasma of mice transplanted with CC-36-FL. As can be seen in Fig. 8, elevated levels of IL-12 ranging from 22 ± 22 to 384 ± 118 pg/ml were observed in plasma at various time points after CC-36-FL transplantation. Plasma samples from normal mice contained only 8 ± 14 pg/ml.

In addition, the levels of IFN-γ were measured in plasma samples of mice transplanted with CC-36-FL. Figure 9 shows the levels of IFN-γ in the plasma of mice transplanted with CC-36-FL. The levels of IFN-γ were elevated 1 day post-CC-36-FL transplantation, and reached a maximum on day 6. The levels of IFN-γ ranged from 15 ± 4 to 126 ± 6 pg/ml of plasma at 1–10-day time-points.

Although the levels of IL-12 and IFN-γ varied during the 9 days follow-up, they were still higher than those in normal mice. Moreover, the elevated levels of IL-12 and

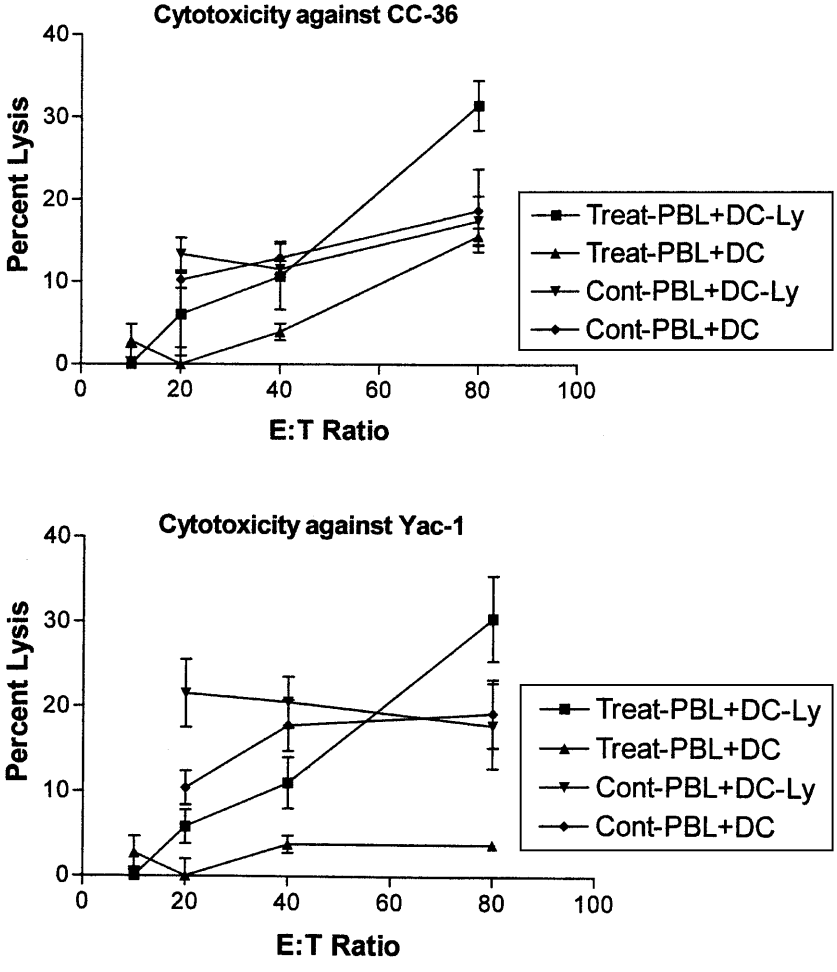

Fig. 7 Cytotoxicity of PBL derived from CC-36-FL + IL-2 treated mice and treatment-free control mice transplanted with parental CC-36 cells. PBL were isolated from the treatment group and the control group. PBL were stimulated with syngeneic DC pulsed with CC-36 lysate and cultured in IL-2 medium for 5 days in a CO_2 incubator. A control culture of PBL with DC alone was used for the treatment group and the control group. A 4-h chromium release assay was performed using the stimulated and cultured PBL as effector cells against the parental CC-36 and the NK-sensitive Yac-1 target cells. Higher CC-36 lysis was observed in PBL from the treatment group (-■-) when compared to the control group (-▼-). However, Yac-1 lysis of PBL from the treatment group (-■-) was similar to the lysis of PBL from the control group (-▼-)

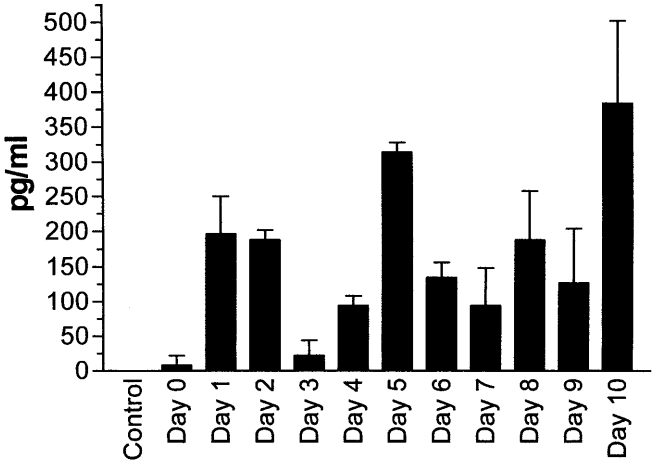

Fig. 8 The levels of IL-12 in the plasma of mice ($n=3$) transplanted with 10^6 CC-36-FL at various time points. The levels of IL-12 were elevated following CC-36-FL inoculation, peaking at day 10 with a level of 384 ± 118 pg/ml. In contrast, the level of IL-12 in the plasma of normal mice was only 8 ± 14 pg/ml

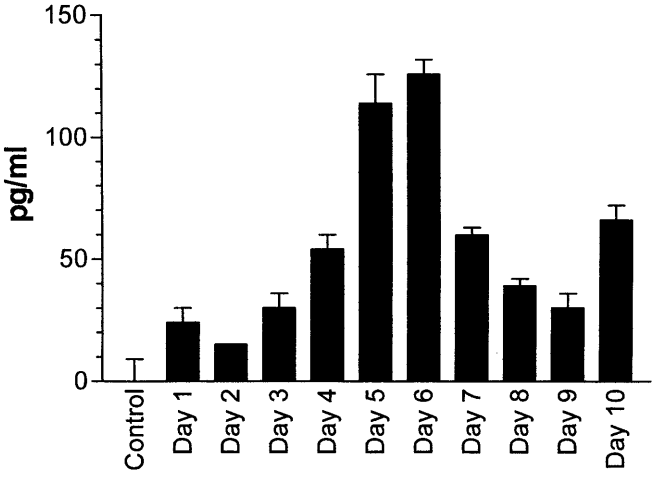

Fig. 9 The levels of IFN-γ in the plasma of mice ($n=3$) transplanted with 10^6 CC-36-FL at various time points. The levels of IFN-γ were elevated (15–126 pg/ml) from day 1 to at least 10 days post-CC-36-FL inoculation. The levels of IFN-γ in the plasma of normal mice were negligible

IFN-γ could have played a partial role in the induction of the observed antitumor effect. However, the tumor burden experiment and survival study showed that the treatment with CC-36FL alone did not produce significant antitumor efficacy. Only the combination of IL-2 andCC-36-FL was able to produce significant antitumor effect by either stimulating specific or non-specific cellular responses, including the CTL, NK and LAK responses.

Discussion

A large number of investigators have reported significant antitumor activity with FL-based immunotherapy. Daily s.c. injections of recombinant soluble FL, alone [2, 4, 6, 9, 11, 21, 25, 32] or with IL-12 [25], IL-2 [21], and CD40 ligand [2], have been used in various murine tumor models with often-dramatic results concerning antitumor immunity. Nevertheless, only two previous reports have demonstrated antitumor activity using a transduced tumor cell line encoding FL [3, 5], yet without combining with other cytokines. The objective of our study was to determine the antitumor efficacy of a colon tumor cell line encoding FL in conjunction with systemic IL-2 in a murine syngeneic colon cancer model. This immunotherapeutic strategy is therefore novel in this regard.

The above experiments with CC-36-FL have demonstrated an increase in circulating DC levels following s.c. injection with irr-CC-36-FL cells, corresponding to increased in vivo FL bioavailability data. It is feasible that the DC produced by the CC-36-FL can infiltrate tumor and stimulate T cells to induce an antitumor response. The tumor burden and survival experiments have shown that a combination of CC-36-FL and systemic IL-2 significantly reduced CC-36 tumor growth and increased the survival of mice transplanted with the CC-36 tumor. This therapy, when compared with that of tumor antigen alone by including irradiated CC-36 cells, tumor antigen plus IL-2 or FL by the administration of CC-36 + IL-2 or CC-36-FL alone produced a significant antitumor effect. These results suggest that a combination of tumor antigens plus FL and IL-2 are important in the induction of an antitumor response. Interestingly, this antitumor synergy between soluble recombinant FL and IL-2 has been noted by other authors [21] in a recently reported sarcoma hepatic metastases model [25].

Moreover, both forms of FL (membrane-bound and soluble proteins) were present in CC-36-FL, which could have induced maximum biological activity since both these forms have been shown to be involved in exerting FL-related antitumor effects [5, 15].

Although the precise mechanism of tumor regression mediated by CC-36-FL + IL-2 therapy remains poorly defined at the present time, we have shown that antigen-stimulated and cultured PBL from the CC-36-FL + IL-2 treated mice lysed the parental CC-36 cells. However, a similar percentage lysis was observed with the NK-sensitive target Yac-1, suggesting the induction of NK activity by this treatment approach. The enhancement of NK activity by this therapy is supported by the fact that the CC-36 FL transplanted mice display elevated levels of IL-12 and IFN-γ in the circulation, which are known to augment NK activity. The precise mechanism responsible for the increased antitumor response in our murine colon cancer model is currently under investigation using CD4-, CD8- or NK-cell-depleted mice.

Other possible antitumor mechanisms may be involved in this combination therapy. Systemic IL-2 administration could enhance the antitumor response of FL-based immunotherapy by means of multiple possible synergistic relationships. Firstly, DC express the IL-2 receptor, and it has been suggested that their migration

into the tissues may indeed be regulated by IL-2 [8]. Vakkila et al. have also demonstrated that DC maintain the viability of IL-2-activated T cells by stimulating the T cells to secrete IL-2, express the high affinity IL-2 receptor, and to proliferate in a mixed lymphocytic reaction [31]. Recently, Shimizu et al. have demonstrated that non-toxic doses of recombinant IL-2 can potentiate the antitumor effects of tumor lysate-pulsed DC in vivo [26, 27]. Lastly, both FL [3, 11, 21, 25] and IL-2 [26] have been reported to increase NK cell antitumor activity. For these reasons, systemic IL-2 could serve as an adjuvant in FL-mediated immunotherapy.

In conclusion, FL expression by our transduced colon tumor cells did not alter in vitro growth nor did it inhibit in vivo tumor formation in athymic nude mice when compared to their wild-type counterparts. Collectively, our results show that IL-2 can potentiate the antitumor effects of therapy with an FL-producing colon tumor cell line in mice with syngeneic colon cancer. The precise synergistic mechanism involved warrants further study; but our data provide a rationale for the use of synergistic cytokines such as IL-2 in FL-based immunotherapy strategies.

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