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ORIGINAL ARTICLE

IL-17E, a proinflammatory cytokine, has antitumor efficacy against several tumor types in vivo

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Abstract Interleukin-17E (IL-17E) belongs to a novel family of cytokines that possess significant homology to IL-17. IL-17E has potent inflammatory effects in vitro and in vivo. Overexpression of IL-17E in mice results in a T helper-2 (Th2)-type immune response, which includes the expansion of eosinophils through the production of IL-5, and elevated gene expression of IL-4 and IL-13 in multiple tissues. In this study, we show that IL-17E has antitumor activity in vivo, a previously unrecognized function of IL-17E. Antitumor efficacy of IL-17E was examined in a variety of human tumor xenograft models, including melanoma, breast, lung, colon, and pancreatic cancers. Injection of recombinant IL-17E every other day resulted in significant antitumor activity in these tumor models. In addition, the combination of IL-17E with chemotherapy or immunotherapy agents showed an enhanced antitumor efficacy in human tumor xenograft models in mice as compared to either agent alone. Antitumor activity was demonstrated using different routes of administration, including intraperitoneal, intravenous, and subcutaneous injection. Anticancer activity was shown for both mouse and human forms of IL-17E, which have a high degree of sequence identity. Tumor-bearing mice treated with IL-17E showed a significant increase in serum levels of IL-5 and increased numbers of eosinophils in peripheral blood compared to the control group. Spleens isolated from IL-17E-treated mice showed a significant increase in eosinophils that correlated with antitumor activity of IL-17E in a dose–response manner. Finally, we demonstrate that B cells are necessary for IL-17E-mediated antitumor activity and that IL-17E was found to activate signaling pathways in B cells in vitro. Taken together, these data demonstrate that IL-17E has antitumor activity in vivo, and support further investigation of the potential clinical use of IL-17E as an anticancer agent.

Keywords IL-17E · Cancer · Antitumor · Eosinophils · B-cells

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Introduction

IL-17E (IL-25) belongs to a novel family of cytokines that possess significant homology to IL-17 [1, 2]. Although IL-17E is structurally related to IL-17 (IL-17A), its biological effects differ from those described for IL-17A and other cytokines in the IL-17 family [3]. The most well-characterized cytokines in this family are IL-17A and IL-17F, both of which induce recruitment of neutrophils and provide protection against extracellular pathogens [3–5].

IL-17A is also important in development and pathogenesis of several autoimmune diseases [4]. In contrast, IL-17E promotes eosinophilia and stimulates the production of T helper 2 (Th2)-type cytokines [5]. The administration of recombinant IL-17E in mice resulted in the expansion of eosinophils through the production of IL-5 from an accessory cell population [2, 7]. In addition, IL-17E induced elevated gene expression of IL-4 and IL-13 in multiple tissues, resulting in a Th2-type immune response characterized by hyperproduction of serum immunoglobulins IgA and IgE, overproduction of mucus, epithelial cell hyperplasia and eosinophilia, indicating that IL-17E is capable of amplifying allergic inflammation [2, 6, 7].

IL-17E is produced by activated Th2 cells and mast cells [7, 8]. IL-17E is also constitutively expressed by CD4+ and CD8+T cells in the gut of mouse strains that are resistant to the helminth *Trichuris muris* [9]. IL-17E was found to have a dual function in helminth infection, first in promoting type 2 cytokine-dependent immunity to gastrointestinal helminth infection, and second in limiting proinflammatory cytokine production and chronic intestinal inflammation by inhibiting expression of type 1 cytokines [9].

Effective tumor surveillance and clearance in mammalian systems require the combined action of both innate and acquired immune responses. Virulizin®, a novel biological response modifier obtained from bovine bile by a standardized extraction process, has demonstrated strong antitumor efficacy in a variety of human tumor xenograft models, including pancreatic cancer, melanoma, breast cancer, ovarian cancer, and prostate cancer [10–13]. Our previous studies have demonstrated a significant role for macrophages and NK cells in the antitumor mechanism of Virulizin® [12, 13]. We recently demonstrated that Virulizin® treatment induced the expression of IL-17E in a melanoma xenograft mouse model, and that production of IL-17E contributed to the antitumor mechanism of Virulizin® [14]. Antitumor activity of Virulizin® was also associated with the expansion of activated B cells and eosinophilia that correlated with IL-17E induction [14].

In this report, we further investigate the antitumor efficacy of IL-17E as a monotherapy and in combination therapy in multiple tumor models. We also show that eosinophils and B cells are involved in the antitumor mechanism of action of IL-17E.

Materials and methods

Cells and animals

Human melanoma cell line C8161 was a gift from Dr. D. R. Welch (Pennsylvania State University, Hershey, PA, USA) [15]. Cells were grown in RPMI 1640 medium (Wisent Inc., St. Bruno, Canada) with 10% fetal calf serum (FCS) at 37°C under 95% air and 5% CO_2, and maintained with routine media changes. Adherent C8161 cells were passaged by trypsinization with 0.025% trypsin. Human tumor cell lines (H460, HT-29, MDA-MB-435, MIA PaCa-2) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in culture medium and under conditions as recommended by the ATCC. CD-1 athymic nude mice (congenitally athymic and deficient in T cells) [48] and SCID mice (deficient in both B and T cells) [48] were purchased from Charles River (Montreal, Canada). All mice were females, between 6- and 8-week-old, weighing approximately 20–25 g. The mice were maintained in the animal facility at Lorus Therapeutics Inc. Animal protocols were in compliance with the Guide for the Care and Use of Laboratory Animals in Canada.

Reagents and antibodies

Recombinant IL-17E was purchased from R&D Systems (Birmingham, AL, USA), and from Peprotech Inc. (Rocky Hill, New Jersey, NY, USA). Phycoerythrin (PE)-conjugated monoclonal anti-mouse CCR3 antibody (R&D Systems) was used for flow cytometry analysis. Antibodies used for Western blots were as follows: Phospho-Tyrosine Mouse mAb (P-Tyr-100), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (Biodesign International, Saco, ME, USA); Rabbit-anti-mouse IkB alpha, phosphorylated (Ser32) (United States Biological Inc, Swampscott, MA, USA); and secondary horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies (Amersham Biosciences, Piscataway, NJ, USA). Antibodies were diluted for use according to the manufacturer's recommendations.

Tumor xenografts and in vivo antitumor efficacy

Human tumor xenografts were established in mice as described [10]. Briefly, tumor cells were cultured to approximately 80% confluence in culture medium and were harvested and suspended in sterile phosphate-buffered saline. Tumor cells (5×10^6 C8161 and H460 cells, 3×10^6 HT-29 cells, 8×10^6 MDA-MB-435 cells, 10^7 MIA PaCa-2 cells) in 100 µl were implanted subcutaneously into the right flank of each mouse (20–28 g body weight). The animals were monitored on a daily basis. When the tumors reached a volume of 50–100 mm^3, mice were randomly separated into groups of ten animals for treatment. For in vivo efficacy studies, mice were treated with recombinant mouse IL-17E or human IL-17E by subcutaneous, intraperitoneal, or intravenous injections as described in the text. For efficacy studies in combination with anticancer agents, mice were treated with human

IL-17E along with the anticancer drugs Cisplatin (Faulding, Montreal, Canada), CPT-11 (Camptosar) (Pharmacia & Upjohn Inc., Peapack, NJ, USA), Taxol (Bristol-Myers Squibb, Montreal, Canada), Tarceva (erlotinib) (Roche Canada, Mississauga, Canada), DTIC (Faulding, Montreal, Canada), Taxotere (Aventis Pharmaceuticals, Bridgewater, NJ, USA), Gemzar (gemcitabine) (Eli Lilly, Indianapolis, IN, USA), or anti-VEGF antibody Avastin (Roche Canada, Mississauga, Canada). All anticancer agents were administered by intravenous bolus injection, with the exception of Tarceva, which was given orally as a solution in PBS. Negative control mice were treated with PBS. Treatment doses, schedules, and routes of administration for the anticancer drugs are described in detail in the text and figure legends. Anti-tumor activity was evaluated as previously described [11]. Tumor volume was estimated by caliper measurements, using the formula: (length × width × height)/2. Tumor weight was determined from tumor tissue surgically excised from the animal on the last day of the experiment. The percent tumor inhibition (%) was calculated using the formula: [(mean tumor weight of control animals − mean tumor weight of drug-treated group)/mean tumor weight of controls] × 100. A P value of ≤ 0.05 was considered to be statistically significant.

Flow cytometry

Spleens were homogenized in PBS containing 2% FCS and filtered with a Cell Strainer (Becton Dickinson, Franklin Lakes, NJ, USA) to produce a cell suspension. Erythrocytes were lysed with ACK solution (0.155 M ammonium chloride, 0.1 mM disodium EDTA, 0.01 M potassium bicarbonate, pH 7.3) for 5 min on ice. For cell surface marker staining, 1×10^6 cells/sample were incubated with PE-conjugated anti-mouse CCR3 antibody in 100 µl of staining solution (PBS containing 2% FCS) on ice for 30 min. The cells were subsequently washed twice with staining solution and fixed with 0.5% paraformaldehyde in PBS. Samples were analyzed by flow cytometry using the FACSCalibur system with CellQuest software (Becton Dickinson, San Jose, CA, USA).

Histochemical detection of eosinophils and computer-assisted image analysis

The excised tumors were fixed in PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate solution) overnight at 4°C. The samples were then dehydrated in graded alcohols, embedded in low melting point paraffin and 5-µm sections were cut on a rotary microtome. Paraffin sections were stained for eosinophils using Sirius red method as described previously [16]. Briefly, the sections were deparaffinized, stained with hematoxylin for 2 s, differentiated in distilled water and treated with 70% ethanol for 2 s, then stained with 0.5% Sirius red (Sigma-Aldrich) solution at room temperature for 1 h. After dehydration with increasing concentrations of ethanol, the sections were mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA).

In vitro stimulation with IL-17E

Cell samples (10^7 cells/ml) in OPTI-MEM medium (Invitrogen Corporation, Carlsbad, CA, USA) were incubated at 37°C and stimulated with human IL-17E (1.4 µg/10^7 cells) for time periods indicated in the text, followed by lysis at 2×10^7 cells/ml in lysis buffer (1% NP40, 150 mM NaCl, 20 mM Tris (pH 7.4), protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na_3VO_4) for 20 min on ice. Nuclei were removed by centrifugation at 16,000g. Protein was quantitated from supernatants with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard.

Western blot analysis

Western blotting was performed as described [17]. Briefly, total protein lysates (40 µg/lane) were resolved on 4–12% SDS-polyacrylamide gels (NuPAGE gels, Invitrogen) and protein transferred to polyvinylidene difluoride (PVDF) membranes. Blots were treated with blocking agent (5% nonfat milk in Tris-buffered saline) for 1 h at room temperature. Protein expression was subsequently detected with primary antibodies diluted according to the manufacturer's recommendations. After washing three times with Tris-buffered saline/Tween 20, a secondary antibody conjugated to HRP at a dilution of 1:10,000 was added and incubated at room temperature for 1 h. The blots were washed, and proteins were detected using an enhanced chemiluminescence detection reagent kit (Amersham Biosciences Inc.) and Kodak X-OMAT AR film for autoradiography.

Serum IL-5 quantitation

Blood was collected from mice and left at room temperature for 4–5 h to coagulate. Serum was separated out by centrifugation at 5,000–7,000 rpm for 5–10 min at 4°C, and supernatant (serum) was transferred into a new tube and frozen at –80°C until needed. Serum IL-5 was measured using an ELISA kit (OptEIA™ Set, BD Biosciences Pharmingen, San Diego, CA, USA) according to manufacturer's instructions. IL-5 values (picograms per milliliter) were calculated by regression analysis using a curve generated by different concentrations of standard preparations of IL-5 (range 1–62.5 pg/ml) diluted in the same buffer.

Statistical analysis

Determination of statistical significance was done using a two-tailed Student's t test. The criterion for statistical significance was $P < 0.05$. Standard error calculations were done using Microsoft Excel.

Results

Antitumor efficacy of mouse IL-17E using different routes of administration

We demonstrated previously that intraperitoneal administration of recombinant mouse IL-17E (mIL-17E) significantly inhibited growth of human melanoma xenografts in a CD-1 nude mouse model [14]. To determine whether mIL-17E has antitumor activity with other routes of administration, we examined the efficacy of mIL-17E following intravenous administration in the same melanoma model. Mouse IL-17E was able to inhibit tumor growth when administered either intraperitoneally or intravenously (Fig. 1a, b). Both routes of administration showed approximately equivalent antitumour efficacy as compared to PBS.

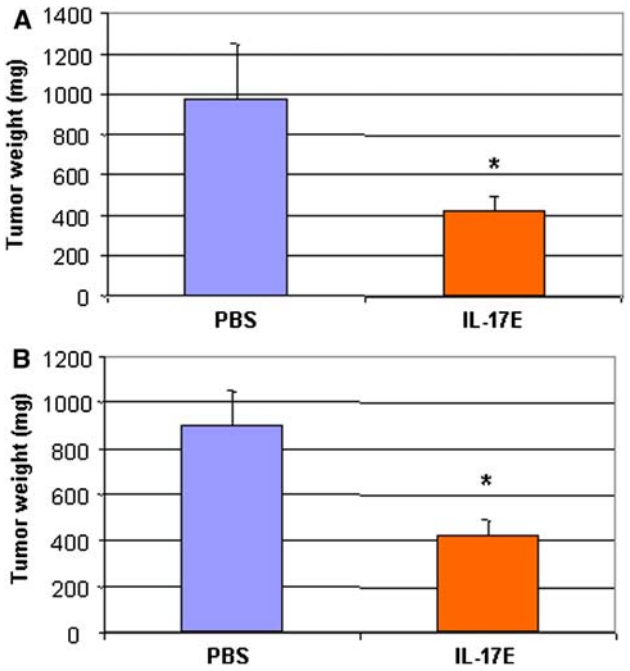

Fig. 1 Antitumor activity of mouse IL-17E given by different routes of administration. Human melanoma C8161 cells were subcutaneously injected into the right flank of CD-1 nude mice. Five days later, the mice were administered with 0.1 ml of PBS or mouse IL-17E (0.04 mg/kg) every 2 days either **a** intraperitoneally (ip) or **b** intravenously (iv) until the end of experiment. Tumors were removed and weights were measured ($n = 10$; $^*P < 0.05$)

Antitumor activity of human IL-17E

Because mouse and human forms of IL-17E share approximately 80% amino acid sequence identity [7] and human IL-17E (hIL-17E) has effector functions in mice including eosinophilia and induction of Th2 cytokines [18], we examined whether hIL-17E might also have antitumor activity. Human IL-17E was tested for antitumor efficacy in a human melanoma xenograft mouse model. We found that hIL-17E demonstrated antitumor efficacy that was roughly equivalent to that of mIL-17E when given by intravenous injection (Fig. 2).

The antitumor activity seen with hIL-17E suggested the potential to develop this cytokine as a cancer therapeutic. Because other cytokines that are in use for cancer treatment are often administered by subcutaneous injection (e.g. Intron A), we explored the anticancer activity of hIL-17E using this route of administration. Initial studies indicated that hIL-17E had antitumor activity when administered by subcutaneous injection in human melanoma xenograft models (Fig. 3a). To expand on this finding, different doses of hIL-17E were administered subcutaneously to CD1 nude mice bearing human melanoma xenografts. Three dose levels were tested to examine the dose–response relationship. The data showed that antitumor activity of hIL-17E was proportionate to dose, indicating that tumor growth inhibition of hIL-17E is dose-dependent (Fig. 3b). The maximum subcutaneous dose of IL-17E tested was 0.144 mg/kg (approximately 3.6 μg per mouse). Higher doses of IL-17E were not tested due to pathological changes to the lungs and digestive tract previously reported in mice given larger amounts (5–10 μg IL-17E every day for 10 days) [7]. Intravenous administration of hIL-17E showed slightly greater anticancer activity than subcutaneous administration using equal doses (0.048 mg/kg every 2 days; data not shown), with no apparent toxicity. Because of this, intravenous administration of IL-17E with

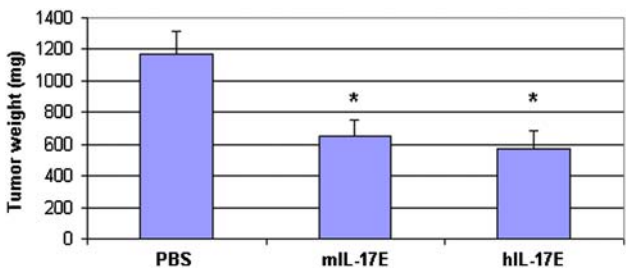

Fig. 2 Comparison of antitumor activities of mouse and human IL-17E in vivo. Human melanoma C8161 cells were subcutaneously injected into the right flank of CD-1 nude mice. Five days later, the mice were administered with 0.1 ml of PBS, mouse IL-17E (0.04 mg/kg), or human IL-17E (0.04 mg/kg) every 2 days until end of experiment. Tumors were removed and weights were recorded ($n = 10$; $^*P < 0.05$)

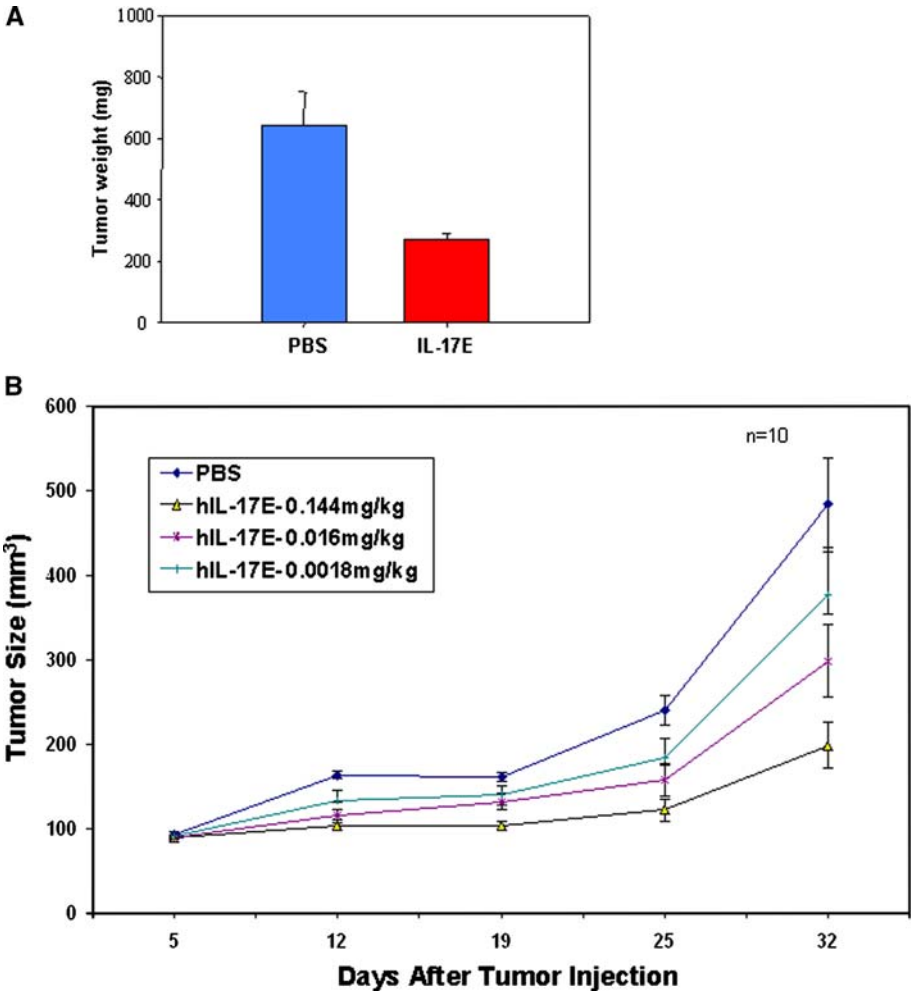

Fig. 3 Antitumor activity of human IL-17E with subcutaneous administration. **a** Human melanoma C8161 cells were subcutaneously injected into the right flank of CD-1 nude mice. Five days later, hIL-17E was administered subcutaneously on the opposite flank into groups of mice ($n = 10$) at 0.04 mg/kg every 2 days for 4 weeks. At the end of the study, tumors were excised and weighed. **b** Dose–response antitumor activity of IL-17E. C8161 tumor-bearing CD-1 nude mice were injected subcutaneously into the opposite flank with IL-17E (0.144, 0.016, and 0.0018 mg/kg) or PBS every 2 days until the endpoint of experiment. At each timepoint, tumors were measured using calipers, and sizes were calculated. Each dose of hIL-17E was diluted in a final volume of 100 ul PBS for injection. A control group of mice were treated with 100 ul PBS. Standard errors of mean tumor volumes are shown

this dosing schedule was used for subsequent in vivo experiments.

Human IL-17E exhibits antitumor efficacy in several human tumor types

We examined the effect of human IL-17E on a variety of xenograft tumor models alone or in combination with drug therapies approved for use in their respective cancer indications. CD1 nude mice bearing the human colon adenocarcinoma HT-29 were treated with hIL-17E, CPT-11, Avastin, or combinations of CPT-11 and IL-17E or Avastin and IL-17E (Fig. 4a). Tumor growth was significantly inhibited by IL-17E treatment, more so than CPT-11 or Avastin alone (Fig. 4a). However, the combination of IL-17E with either chemotherapeutic drug was most effective in reducing tumor growth. These results suggest that IL-17E treatment either alone or in combination therapy may be advantageous for the treatment of colon adenocarcinoma.

Similar efficacy results were obtained in four other tumor types tested (non-small cell lung cancer, melanoma, breast adenocarcinoma, and pancreatic cancer). In each of these models, IL-17E alone showed significant antitumor efficacy relative to the PBS control group. Furthermore, the combination of IL-17E with each cancer therapeutic tested was greater than either agent alone. Tumor regressions with IL-17E combination therapies were observed in melanoma (Fig. 4c) and pancreatic cancer (Fig. 4e). IL-17E alone was particularly effective against breast and non-small cell lung cancer, with tumor volumes and weights decreased by approximately 80% compared to PBS control groups (Fig. 4b, d). Tumor weight measurements confirmed significant decrease in tumor sizes for all five tumor types (Fig. 5a–e). No significant loss of body weight was observed with the IL-17E dose used for these studies (data not shown), suggesting that IL-17E toxicity is low at efficacious doses.

Antitumor mechanism of IL-17E requires B cells

To elucidate the anticancer mechanism of action of IL-17E, we first examined the effect of B cells on IL-17E antitumor activity. Earlier studies have shown that overexpression of IL-17E in transgenic mouse models results in increased B cells in the peripheral blood, spleen and lymph nodes of

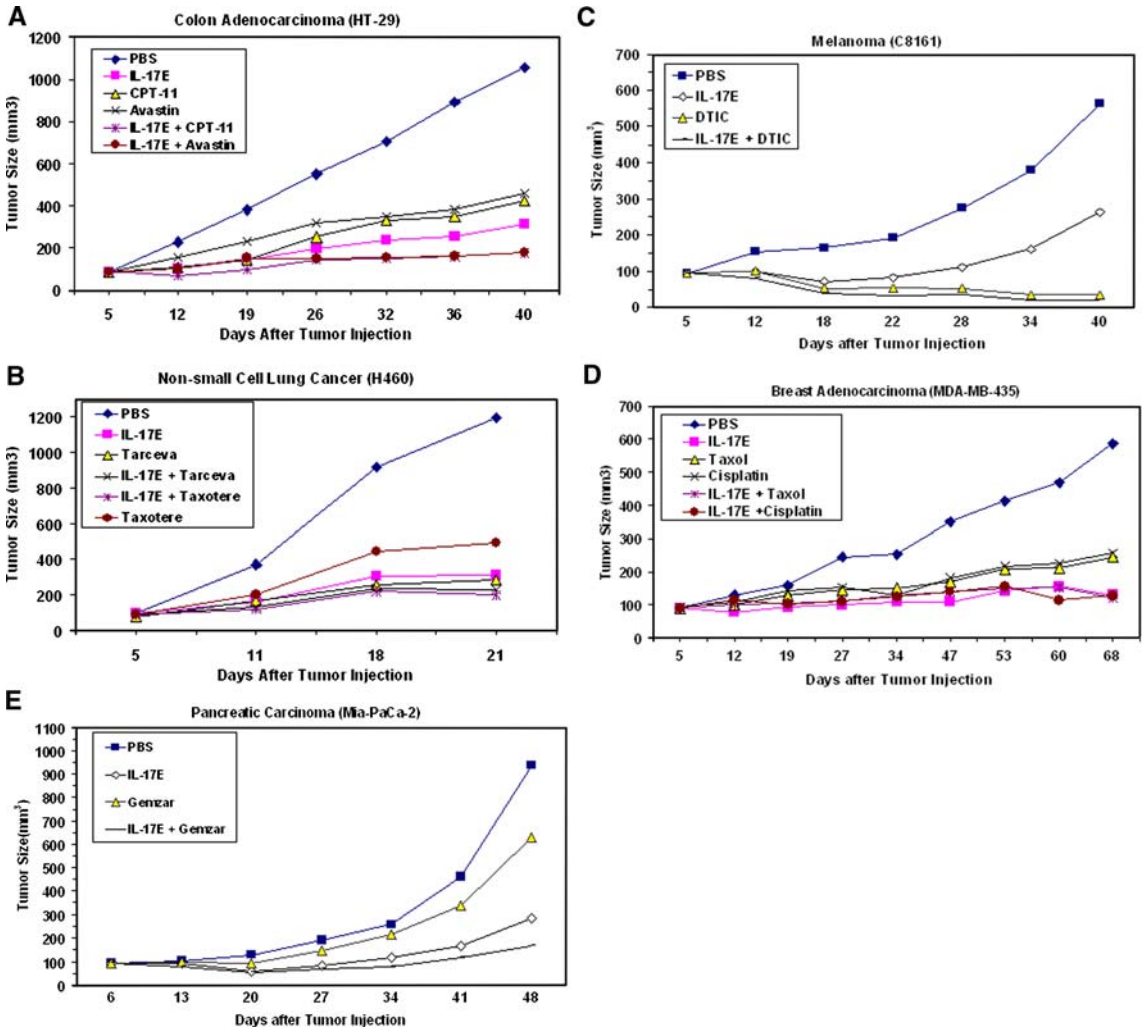

Fig. 4 Antitumor activity of human IL-17E against various human tumors, alone and in combination with cancer therapeutics. **a**–**e** Plots of sizes of tumor xenografts in CD-1 mouse models over the course of the study. Tumor sizes (mm^3) were measured at each point shown in the growth curves. For each study, hIL-17E was administered by intravenous (i.v.) injection at 0.04 mg/kg every 2 days. Doses and routes of administration for cancer therapeutics are shown below. Control groups were given 100 ul PBS by i.v. injection every 2 days. All treatments were administered for the duration of the study. For combination studies, each agent was administered at the same dose level used for single-agent experiments. **a** Human colon adenocarcinoma (HT-29). Mice ($n = 10$ per group) were treated with hIL-17E, CPT-11 (20 mg/kg every 5 days, i.v.), Avastin (0.4 mg/kg every 2 days, i.v.), or combinations of CPT-11 and IL-17E or Avastin and IL-17E. **b** Human non-small cell lung carcinoma (NSCLC) (H460). Mice were treated with hIL-17E, Tarceva (100 mg/kg in 200 µl of vehicle solution, one time per day, oral), Taxotere (10 mg/kg in 100 µl of vehicle solution, one time per week, i.v.), or combinations of IL-17E and Tarceva or IL-17E and Taxotere. **c** Human melanoma (C8161). Mice were treated with hIL-17E, DTIC (80 mg/kg per mouse, one time per week, i.p) or a combination of IL-17E + DTIC. **d** Human breast adenocarcinoma (MDA-MB-435). Mice were treated with hIL-17E, Taxol (10 mg/kg in 100 µl of vehicle solution, one time per week, i.v.), cisplatin (4 mg/kg in 100 µl of vehicle solution, one time per week, i.v.), or combinations of IL-17E and Taxol or IL-17E and cisplatin. **e** Human pancreatic carcinoma (MIA PaCa-2). Mice were treated with hIL-17E, gemcitabine (100 mg/kg, one time per week, i.v.) or a combination of both agents. Standard error measurements for each data point did not exceed 25% of the mean tumor volume

transgenic mice [18], suggesting that B cell proliferation is stimulated by IL-17E. We examined the role of B cells in IL-17E anticancer activity by testing the effect of IL-17E treatment on C8161 xenografts in SCID mice (B and T cell deficient) versus CD-1 nude mice (T cell deficient). While IL-17E treatment reduced tumor growth in CD1 nude mice compared to PBS controls, IL-17E did not have a significant effect on tumor growth in SCID mice (Fig. 6a, b). These results suggest that B cells are required for the antitumor mechanism of IL-17E.

Eosinophils are increased upon IL-17E treatment

IL-17E has been reported to increase eosinophils, both in transgenic mouse models [18] and also upon treatment of wildtype (C57BL/6) mice with recombinant IL-17E [7]. To

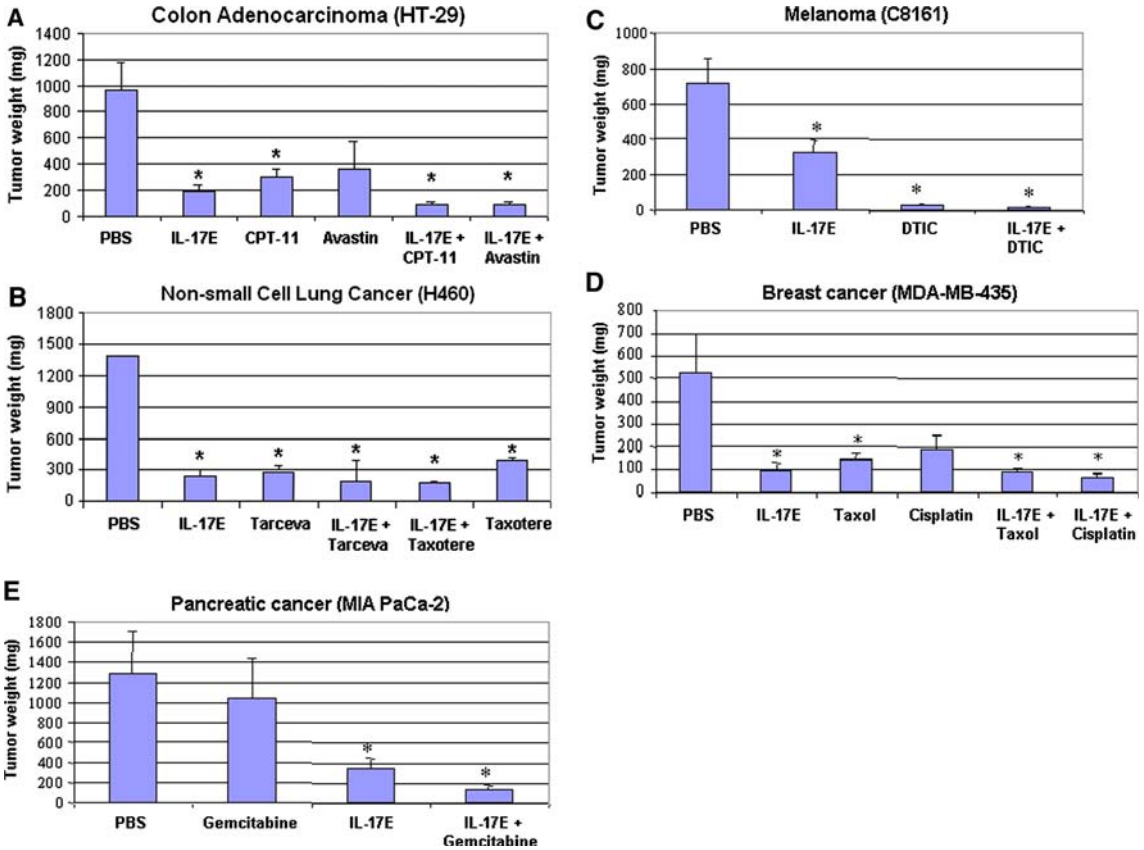

Fig. 5 Tumor weight measurements from efficacy studies with IL-17E alone and in combination with various anticancer drugs. At the end of the in vivo efficacy studies (described in Fig. 4), tumors were excised and weighed. Tumor weights for colon cancer, NSCLC, melanoma, breast cancer, and pancreatic cancer models showed a statistically significant reduction in tumor weights ($*P < 0.05$) compared to those from PBS-treated mice (**a**–**e**)

determine whether eosinophils were increased in our studies in tumor-bearing CD-1 nude mice, eosinophils in blood and spleen from IL-17E-treated mice were analyzed by flow cytometry using the surface marker for mouse eotaxin receptor, CCR3, which has been shown to be expressed exclusively on mouse eosinophils [26]. The results showed that blood and spleens from IL-17E-treated mice had higher percentages of CCR3+ cells compared to controls (Fig. 7a, b). Levels of CCR3+ cells in spleens correlated with antitumor activity of hIL-17E in a dose–response manner (Fig. 7c). SCID mice treated with IL-17E, in which tumor growth was unaltered by IL-17E treatment, only demonstrated a minor increase in splenic eosinophils as compared to PBS controls (data not shown). These results suggested that eosinophil infiltration into the periphery might play a role in the antitumor mechanism of IL-17E.

To expand on this finding, we examined the tumors of IL-17E-treated mice for infiltration of eosinophils into tumors. Numerous studies have demonstrated a role for eosinophils in tumor immunity (reviewed in [25]), suggesting that eosinophils may also be involved in the antitumor activity of IL-17E. Mice with C8161 tumors were injected intravenously with either PBS or hIL-17E. Tumors were then excised from mice and eosinophil infiltration of tumors was examined by histochemistry studies. Paraffin sections were prepared and stained for eosinophils using Sirius red. Results revealed that there was indeed an increase in eosinophil infiltration into tumors isolated from IL-17E-treated mice as compared with controls (Fig. 8a, b). Quantitative image analysis of data obtained from six tumor samples per group demonstrated that the average number of eosinophils per field was approximately fivefold greater in the IL-17E-treated group as compared to the control group, and that eosinophil infiltration was significantly elevated in tumors following IL-17E treatment ($P < 0.01$) (Fig. 8a).

Serum IL-5 is increased upon IL-17E treatment in vivo

Previous studies have shown that blood eosinophilia resulting from treatment with IL-17E is mediated by the

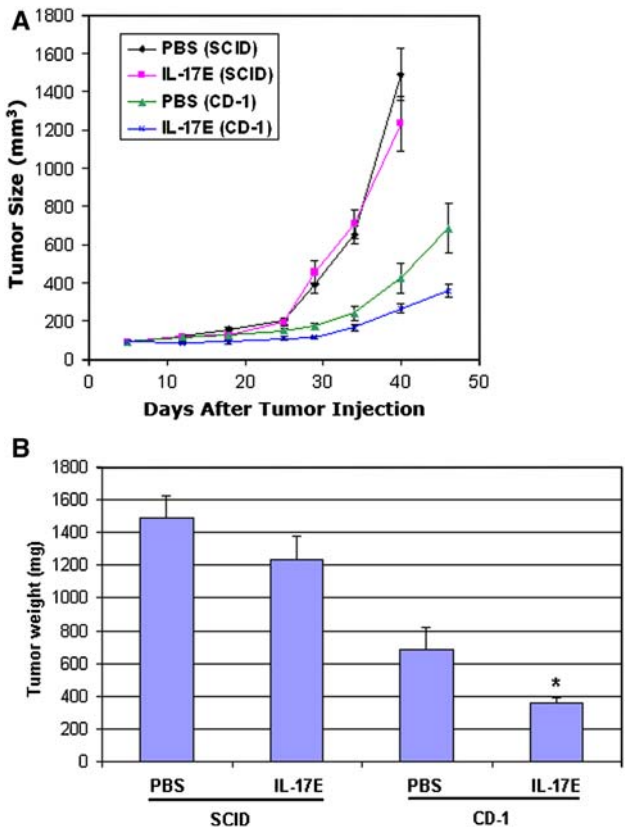

Fig. 6 B cells are required for IL-17E antitumor activity. Human melanoma C8161 cells were implanted subcutaneously in the right flank of SCID and CD1 nude mice. Five days later, mice were treated intravenously with either 0.1 ml of PBS or human IL-17E (0.04 mg/kg) every 2 days for the duration of the experiment. At each timepoint, tumors were measured using calipers, and sizes were calculated and plotted (**a**). At the end of the study tumors were excised and weighed (**b**). Tumors from SCID mice treated with IL-17E were not significantly reduced compared to tumors from PBS-treated SCID mice ($P = 0.4$), whereas tumors from IL-17E-treated CD-1 mice were significantly smaller relative to control ($^{*}P = 0.04$ relative to PBS control)

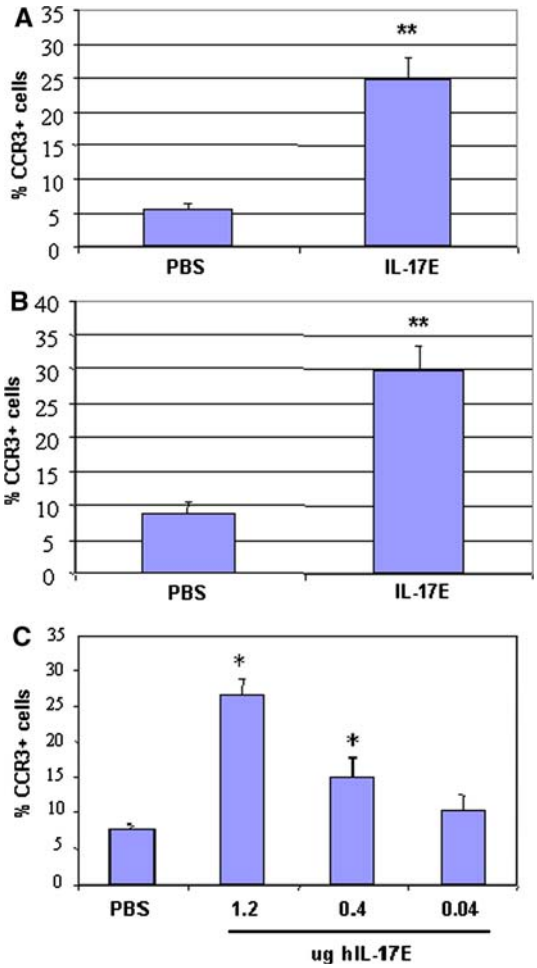

Fig. 7 IL-17E stimulates eosinophil numbers in blood and spleen. CD-1 nude mice bearing C8161 human xenografts were given intraperitoneal injections of either 0.1 ml PBS or IL-17E (0.04 mg/kg). Blood (**a**) and spleens (**b**) were collected, red blood cells were removed, and cells were analyzed for eosinophils by flow cytometry using anti-CCR3-PE antibodies. **c** Splenic eosinophils in spleens from tumor-bearing mice treated with increasing doses of IL-17E. CD-1 nude mice with C8161 xenografts were treated with subcutaneous injections of PBS (0.1 ml) or human IL-17E (1.2, 0.4, and 0.04 μg per mouse; equivalent to 0.048, 0.016, and 0.0018 mg/kg, respectively) every 2 days for 4 weeks. CCR3+ cells were quantitated by flow cytometry as described above. Percentages of CCR3+ cells in the granulocyte population are shown in the *graphs* ($^{**}P < 0.01$ compared to PBS control)

induction of IL-5 [7]. IL-5 is a Th2 cytokine that regulates eosinophil development and function, and which recruits eosinophils from the bone marrow into tissue through the blood [19]. To determine whether IL-5 was induced in our tumor efficacy models, serum IL-5 levels were examined from tumor-bearing CD-1 nude mice that were treated with human IL-17E. Levels of IL-5 were significantly increased in serum from IL-17E-treated mice in two different tumor models: HT-29 and C8161 (Fig. 9a, b). Serum levels of IL-5 showed a dose–response effect with increasing doses of hIL-17E (Fig. 9b). The induction of IL-5 by IL-17E correlated with the antitumor effect (Fig. 3b) and increased splenic eosinophils (Fig. 7c) seen with the same doses in this tumor model. These results suggest that secretion of IL-5 by IL-17E treatment promoted the influx of eosinophils into the spleen, and that IL-5 contributes to the antitumor mechanism of action of IL-17E.

IL-17E activates signaling pathways in B cells

Since B cells were required for antitumor activity in response to IL-17E treatment (Fig. 6), we examined the effect of IL-17E on signaling pathways involved in activation of B cells. For these studies, we used the mouse B cell lymphoma cell line WEHI-231, which is known to

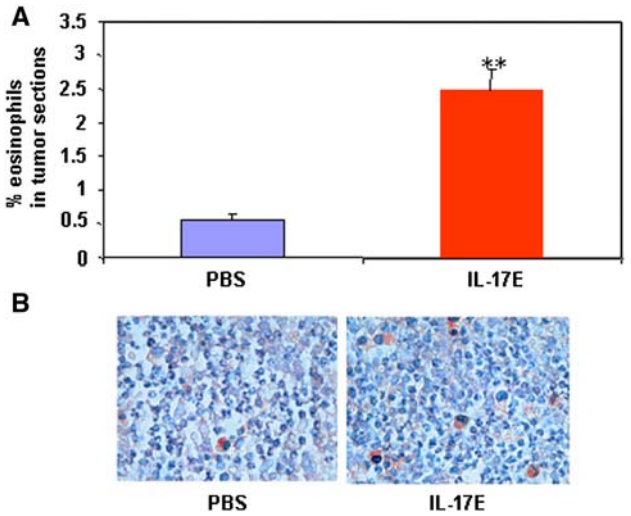

Fig. 8 Increased infiltration of eosinophils into tumors with IL-17E treatment. C8161 tumors from IL-17E-treated mice were collected and paraffin sections of tumors were prepared and stained for eosinophils using Sirius Red method. The number of eosinophils per square millimeter of tumor tissue (**a**) was determined by computer-assisted image analysis. Each *bar* in the graph represents the mean ± SE of determinations in six samples of the same treatment (**$P < 0.05$ compared to PBS control). **b** Representative micrographs of sections show stained eosinophils in tumors from mice treated with PBS or IL-17E

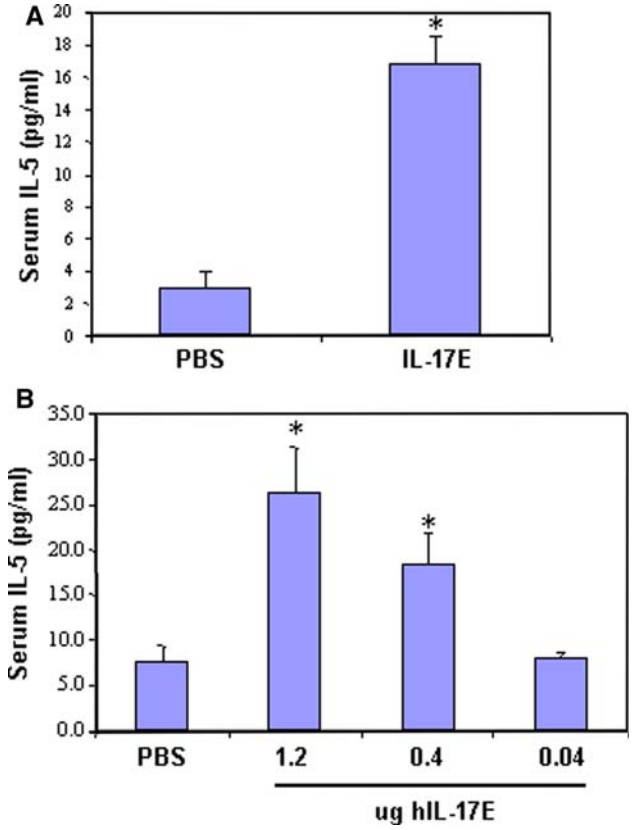

Fig. 9 Treatment of tumor-bearing mice with IL-17E results in induction of serum IL-5. Serum was collected from CD-1 nude mice engrafted with human tumors. **a** HT-29 human colon adenocarcinoma. Mice were treated with intravenous injections of either PBS (0.1 ml) or human IL-17E (0.04 mg/kg) every 2 days for 4 weeks. **b** C8161 human melanoma. Mice were treated with subcutaneous injections of PBS (0.1 ml) or human IL-17E (1.2, 0.4, and 0.04 μg per mouse) every 2 days for 4 weeks. IL-5 was quantitated in serum samples using an ELISA method. Results are expressed in pg IL-5/ml of serum

express the receptor for IL-17E [20]. WEHI-231 cells were exposed to both mouse and human IL-17E for various time periods in vitro, and cell lysates were probed for changes in tyrosine phosphorylation. The results revealed that both forms of IL-17E were able to induce tyrosine phosphorylation of cellular proteins, primarily those between 50 and 75 kDa (Fig. 10a, b). The strongest increase in phosphorylation occurred with exposure of cells to IL-17E for 2–5 min, and returned to baseline by 30 min.

Both human and mouse IL-17E can activate the NF-kappaB pathway in B cells

IL-17E has been shown to activate NF-kappaB in renal cell carcinoma cells [27], human eosinophils [45], and mammalian cells overexpressing mouse IL-17E receptor [46]. To determine whether IL-17E activates NF-kappaB in B cells, we incubated WEHI-231 cells with human or mouse IL-17E. NF-kappaB activation was examined by analyzing phosphorylation of IkappaB-α. Figure 11 shows that untreated cells expressed low levels of phospho-IkappaB-α. Cells exposed to either human or mouse IL-17E showed increased phosphorylation of IkappaB-α indicative of activation of the NF-kappaB pathway. Activation occurred rapidly, as levels of phospho-IkappaB-α increased within 2 min, then returned to baseline levels within 5 min. Both mIL-17E and hIL-17E activated NF-kappaB equally.

Discussion

IL-17E was originally identified based on sequence similarity to other members of the human IL-17 family (IL-17, IL-17B, and IL-17C) [27]. Purified recombinant hIL-17E was also shown to have similar effector functions as IL-17, including induction of expression of NF-kappaB and the pro-inflammatory cytokine IL-8 [27]. Since then several studies have demonstrated that IL-17E (IL-25) mediates a number of biological activities that are distinct from those of other IL-17-related cytokines, including Th2-type immune responses and eosinophilia [6, 7, 18]. Preliminary studies conducted by our research group suggested that IL-17E also had antitumor activity, based on data showing that intraperitoneal injections of mouse IL-17E decreased the growth of human melanoma tumor xenografts in mice [14]. The present study further examines the antitumor efficacy of both human and mouse recombinant IL-17E in a variety

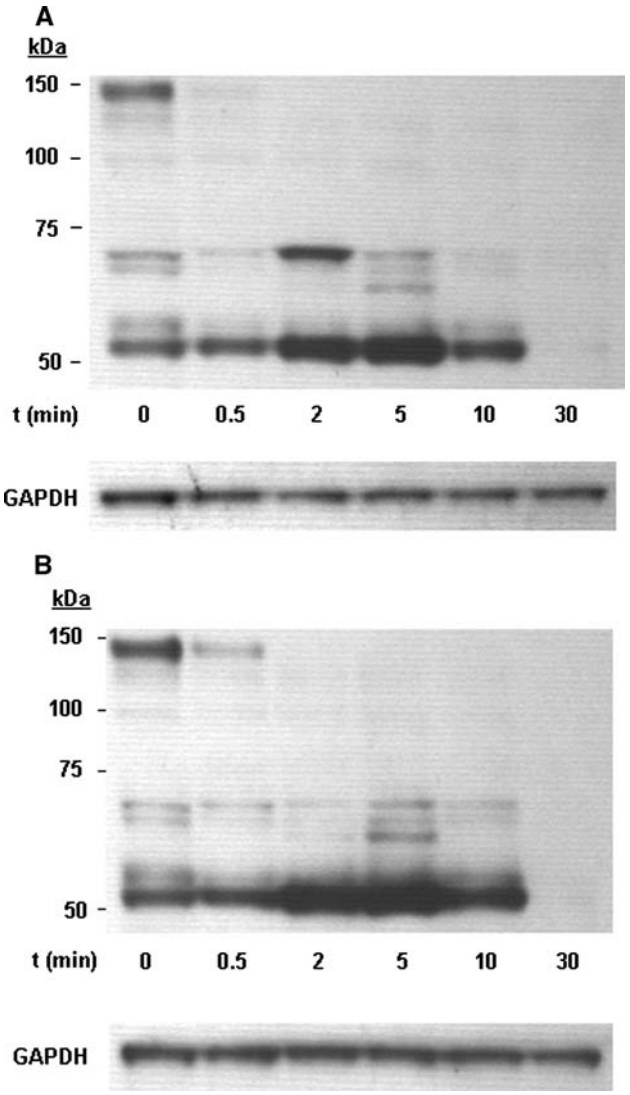

Fig. 10 IL-17E activates signaling pathways in B cells. WEHI-231 cells were stimulated in vitro (10^7/ml) for $t = 0$–30 min with 1.4 μg/ml of either human IL-17E or mouse IL-17E at 37°C. After cell lysis, 25 μg of protein lysate was loaded onto 4–12% SDS-PAGE gels followed by Western blotting using anti-phosphotyrosine antibody and HRP-conjugated anti-mouse-antibody. Blots were stripped and probed for GAPDH as a loading control. Molecular weight markers in kilodaltons (kDa) are indicated. A similar phosphorylation pattern was noted for both human IL-17E (**a**) and mouse IL-17E (**b**)

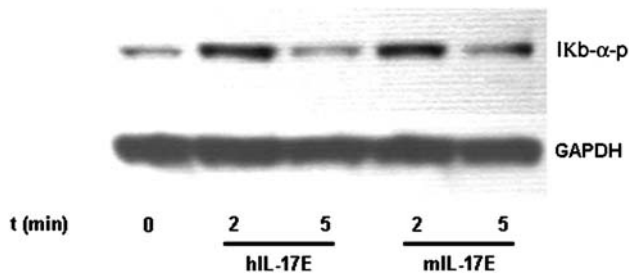

Fig. 11 Human and mouse IL-17E can activate NF-kappaB in B cells. **a** WEHI-231 cells (10^7/ml) were stimulated in vitro with 0.7 μg/ml of either hIL-17E or mIL-17E for $t = 0$, 2 or 5 min at 37°C. Cells were lysed in lysis buffer for 30 min on ice, followed by centrifugation for 10 min. After cell lysis, 30 μg of protein was separated onto 4–12% SDS-PAGE gels, followed by Western blotting with anti-phospho-IkB-alpha antibodies, followed by HRP-conjugated anti-rabbit antibodies, and developed using chemiluminescence. Blots were then stripped and reprobed with GAPDH together with HRP-conjugated anti-mouse antibodies

of human tumor xenograft models. Human IL-17E showed significant antitumor activity against human melanoma, pancreatic, colon, lung, and breast tumor models. Furthermore, in comparison with the efficacies of standard chemotherapeutic drugs administered concurrently, the anti-tumor activity of IL-17E was equal or superior to Avastin, CPT-11, Gemzar, Tarceva, Taxotere, Cisplatin, and Taxol. These studies suggest that hIL-17E may have potential for use as a cancer treatment. This is supported by the low toxicity of hIL-17E at efficacious doses used in our animal studies, suggesting that IL-17E may have an acceptable therapeutic index for cancer therapy.

Our results suggest that the antitumor mechanism of IL-17E requires B cells since tumors were not reduced in SCID mice treated with IL-17E. Tumor growth was inhibited by IL-17E treatment in athymic CD1-nude mice, which lack T cells, indicating that T cells are not necessary for the antitumor effects of IL-17E. This is in contrast to parasitic infection, whereby neither T nor B cells are obligatory for *N. brasiliensis* expulsion after IL-25 treatment [22]. Previous studies have reported that the mouse IL-17E receptor is expressed in some B cell lines [20]. Our results demonstrate that one of these B cell lines, WEHI-231, responds in vitro to IL-17E by induction of tyrosine phosphorylation. Preliminary data suggest that some of the phosphorylated proteins activated by IL-17E are involved in MAPK signaling pathways in B cells (data not shown). Interestingly, IL-17E has previously been shown to stimulate mouse splenocytes in vitro, resulting in expression of IL-5 [28]. This is consistent with our findings that IL-17E activates splenic B cells. Fort et al. [7] reported that IL-17E induces non-T/non-B MHC class II ^{high} expressing accessory cells to produce IL-5. Whether these accessory cells are also induced in our system to secrete IL-5 by some cytokine released by B cells activated through IL-17E remains to be determined. Although we found that serum IL-5 was increased with IL-17E treatment, B cells did not show significant induction of IL-5 (data not shown).

The mechanisms of tumor inhibition by IL-17E treatment are still being explored. Our results demonstrated that IL-17E treatment in vivo leads to eosinophilia in the periphery of CD1 nude mice. Eosinophilia correlated with tumor inhibition, since SCID mice treated with IL-17E not only exhibited lack of tumor inhibition but also a significant decrease in splenic eosinophilia. These results

underscore the connection between the presence of B cells and eosinophil infiltration. Eosinophilia induced by IL-17E is due to induction of the cytokine IL-5 [2, 7]. IL-5 has several effector functions in eosinophils, including eosinophil proliferation and maturation, and is a survival factor for eosinophils by delaying apoptosis [29]. We found that upon IL-17E treatment in vivo, both serum IL-5 and splenic eosinophils were induced in a dose-dependent manner, correlating with an antitumor response. In our earlier study, we showed that administration of the anticancer agent Virulizin increased serum levels of IL-17E and IL-5, and infiltration of eosinophils into tumors [14]. The results of the present study suggest that IL-5 and eosinophils are important mediators of the antitumor mechanism of IL-17E.

Numerous studies have shown that eosinophils are potent effector cells that have direct and indirect tumor killing activities in vitro and in vivo [25]. Rejection of syngeneic lymphoma and melanoma tumors in mice was associated with heavy infiltrations of eosinophils following injection of tumors with mouse dendritic cells and natural killer/lymphokine-activated cells [47]. Additional evidence of this has been derived from transgenic mouse models, in which IL-5 overexpressing transgenic mice showed decreased incidence and growth of methylcholanthrene (MCA)-induced fibrosarcomas, with high level of eosinophils within and surrounding the tumors [30]. As well, eosinophil-deficient transgenic mouse strains had significantly increased incidence of MCA-induced tumors compared to syngeneic wild type controls [30]. Further evidence of an anticancer role for eosinophils comes from reports that correlate eosinophil infiltration in tumors with positive clinical outcome, including patient survival in colorectal cancer [31, 32] and decreased lymph node metastasis in esophageal squamous cell carcinoma [33]. In contrast, eosinophils may play a role in promoting tumor progression by inducing tumor angiogenesis [23]. Eosinophils produce pro-angiogenic factors, including VEGF and bFGF, and lysates of human eosinophils can induce VEGF mRNA expression in endothelial cells [49]. In addition, eosinophils have been shown to induce endothelial cell proliferation and blood vessel growth in a variety of angiogenesis models, convincingly demonstrating that eosinophils have pro-angiogenic functions, supporting the role of these cells in promoting inflammation and allergic diseases [49, 50]. The potential role of eosinophils on angiogenesis in our tumor models has not been determined. Intriguingly, inhibition of tumor progression has been associated with high levels of tumor eosinophils and IL-5 overexpression in mouse tumor models without significant changes in tumor vasculature [30]. This observation, combined with our results showing that IL-17E treatment results in dose-dependent serum IL-5 induction and tumor eosinophil recruitment, support the suggestion that IL-5-activated eosinophils in tumors may have anti-tumor functions [23, 30].

We demonstrated significant antitumor activity with IL-17E in a nude mouse model, indicating that IL-17E has an anticancer effect in a T cell-deficient environment. IL-17E is expressed in CD4+ and CD8+ cells but not other innate immune cells in the gut of parasite-resistant mice, suggesting that an effector T cell population exists that selectively produces IL-17E [9]. IL-17E not only enhances IL-4 production and subsequent amplification of Th2-mediated immune responses but also controls inflammation during helminth infection by dampening Th1 responses [9]. IL-17E-secreting T cells may belong to a distinct lineage separate from Th2 cells and may be subject to different modes of regulation, as recently suggested [21]. Understanding how to differentiate and/or activate such cells in the future may be instrumental in providing the proper network of Th cells to help aid in tumor destruction. Regulation of IL-17E expression appears to be crucial for Th2 responses in allergic diseases [5], although the mechanisms of regulation are not well understood. Factors that affect IL-17E function may be equally important. Recently, IL-17E (IL-25)-mediated induction of robust Th2 responses was shown to be profoundly influenced by matrix metalloproteinase 7 (MMP7) [24]. MMP7-cleaved recombinant mouse IL-17E showed enhanced binding to IL-17E receptor B-Fc fusion protein compared to native rIL-17E, and also induced significantly higher levels of Th2 cytokines in naïve mouse lymph node cells and spleen cells in vitro [24]. Compared to wild-type mice, lungs of $MMP7^{-/-}$ mice showed reduced eosinophilia and lower concentrations of IL-4 and IL-5 upon nasal administration of rIL-17E, as well as reduced expression of IL-17E following challenge with allergen in an experimental asthma model [24]. These findings demonstrate that MMP7 is essential for Th2-responsiveness of IL-17E in allergic inflammation, suggesting the possibility that MMP7 may also impact the antitumor activity of IL-17E.

The ability of IL-17E to mediate antitumor activity adds a novel function to this unique family of cytokines. Previous studies have suggested a potential role for IL-17A in protective tumor immunity in vivo. Ectopic overexpression of murine IL-17 in mouse hematopoietic tumors significantly inhibited tumor growth in immunocompetent mice by means of a T-cell dependent mechanism [34]. Expression of endogenous IL-17 also contributes to antitumor immunity. Growth and metastasis of murine colon cancer was greatly enhanced in IL-17-deficient mice compared to C57BL/6 wild-type mice, attributed to potential defects in the NK- and T-cell functions in the IL-17-knockout mice [35]. Other studies have implicated the involvement of Th17 cells, which are IL-17-producing T cells of CD4+

lineage (reviewed in [36]). A number of reports have identified Th17 cells in the tumor microenvironment in mouse and human tumors [37–40], and increased levels of Th17 cells in ovarian cancers have been shown to be prognostic for improved patient survival [40]. Furthermore, tumor-specific Th17 cells have induced tumor regression and improved survival upon adoptive transfer in a syngeneic mouse melanoma model [41]. Kryczel et al. [35] reported that recombinant IL-17 has no significant effect on cancer cell proliferation in vitro. Similarly, we have found that IL-17E does not inhibit growth of cancer cells in vitro (data not shown). To the best of our knowledge, the potential role of IL-17E in the context of IL-17 and/or Th17 antitumor activities has not been examined. Interestingly, IL-25 suppresses Th17 function through induction of the Th2 cytokine IL-13, and IL-25 is important in mitigating the inflammatory response mediated by Th17 cells in animal models of experimental autoimmune encephalomyelitis (EAE) [42]. These studies demonstrate how two related cytokines can have antitumor functions while having opposing roles in inflammation. The findings of these reports and the current study also add to the growing evidence of the link between cytokines, inflammation, and cancer progression [43, 44]. Further studies are clearly warranted to examine the intriguing relationship between IL-17 and IL-17E functions and their contributions to disease pathologies.

In summary, administration of IL-17E showed antitumor efficacy in the treatment of human melanoma, pancreatic, lung, colon, and breast cancer xenograft models. Our results demonstrate a previously unknown function of IL-17E as a novel immunotherapeutic agent, and suggest that IL-17E may be an effective anticancer therapy for treatment of a wide variety of tumor types.

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