In-vivo Anti-Cancer Efficacy of Recombinant Mouse IL-17E in Syngeneic Mouse Colon and Melanoma Models in Balb/c and C57BL/6 Mice

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Abstract

Previous work from our laboratory has demonstrated the in-vivo anti-tumor activities of both recombinant mouse and human IL-17Es in multiple human tumor xenograft models including colon and melanoma in immuno-deficient mice. The lack of T-cell function in these animals indicated that the IL-17E-mediated anti-tumor efficacy achieved was attributable to the B cell activation and other immune-effector functions of IL-17E in-vivo. To further investigate the anti-cancer properties of IL-17E and the behavior of tumors in a more compatible tumor microenvironment, the recombinant mouse IL-17E (rmIL-17E) protein was evaluated in syngeneic (CT26) colon and (B16-F10) melanoma models in immuno-competent Balb/c and C57BL/6 mice. Initially, IL-17E receptor expression analysis by Western blotting revealed that both cancer cell lines express the receptor for IL-17E. In-vitro cell based sensitivity (XTT) assays showed that rmIL-17E was able to induce cell growth inhibition and apoptosis in these cell lines after a 24 hour incubation suggesting that direct cytotoxicity, in combination with immuno-modulation in vivo, may contribute to anti-tumor efficacy. Apoptosis was detected in-vitro by PARP cleavage in both cell lines after treatment with rmIL-17E. We also showed in this study that rmIL-17E administered i.v. at non-toxic doses every 2 days was efficacious in-vivo and mediated tumor growth inhibition in a dose-dependent manner in both CT26 and B16-F10 syngeneic models. Subcutaneous CT26 tumors grew more aggressively than B16-F10 in Balb/c mice and required higher doses of rmIL-17E (2.5ug and 5.0 ug per mouse) to achieve significant efficacy with tumor growth inhibition of 54% ($p<0.05$) and 62% ($p<0.05$), respectively. Whereas slow-growing B16-F10 melanoma responded well to rmIL-17E treatment at 1.0ug and 2.5 ug with tumor growth inhibition of 69% ($p<0.05$) and 84% ($p<0.05$), respectively, in the Balb/c model. Re-evaluation of dose response in C57BL mice revealed a more aggressive tumor growth of B16-F10. However, significant efficacy was still achieved with rmIL-17e at 5.0 ug/mouse by i.v. bolus injection every 2 days. Additionally, our B16-F10 Lung Metastasis Model in Balb/c mice showed significant protection against melanoma cancer cell infiltration and tumor growth in the lungs of rmIL-17E treated mice based on reduced number of tumor nodules/ foci counted on the lung surface compared to that of control lungs ($p<0.05$) several days post-tail vein inoculation. In conclusion, the in-vivo syngeneic mouse tumor model presents a useful and flexible system to evaluate potential novel therapeutic such as IL-17E with immuno-modulatory functions in the treatment against cancers.

Introduction

- IL-17E is a novel proinflammatory cytokine that induces a Th2-type immune response, which includes the expansion of eosinophils thorugh the production of IL-5, and elevated gene expression of IL-4 and IL-13 in multiple tissues. [Fort, et al. (2001) Immunity, 15:p.985].
- In addition to immunomodulation, IL-17E has been shown to induce direct cytotoxity to IL-17E receptor expressing cancer cells through apoptosis [Furuta, et al. (2011) Sci.Transl. Med.3:p.1].
- Treatment with IL-17E has achieved anti-tumor efficacy in the human melanoma, pancreatic, lung, colon, and breast cancer xenograft models in nude mice [Benatar, et al.(2010) Cancer Immunol. Immunother. 59: p.805].
- Since our previous studies have been done in nude mice, the potential role of T cells on anti-tumor activity and toxicity of IL-17E in immuno-competent mice is not known. Hence, the development of mouse syngeneic tumor models is reported here.
- Anti-tumor activity of IL-17E is also investigated in a B16-F10 lung metastasis model.
- Here we present the preliminary data relating to IL-17E efficacy in the (CT26) colon and (B16-F10) melanoma mouse syngeneic subcutaneous tumor models and a B16-F10 lung metastasis model to further support the potential clinical use of IL-17E as an anticancer agent. All animal study protocols are approved by the the Animal Care Committee of Lorus Therapeutics, Inc. in accordance with the Canadian Council on Animal Care.

Summary of Results

- **rmIL-17E was efficacious in-vivo in both the mouse CT26 and B16-F10 syngeneic tumor models and mediated significant anti-tumor activity in a dose-dependent manner.**
- **rmIL-17E was capable of inducing significant cell growth inhibition and apoptosis in these two cell lines in-vitro.**
- **rmIL-17E showed significant anti-metastatic activity in our B16-F10 lung metastasis model resulting in a reduced number of tumor nodules/foci in the lungs of treated mice compared to the control.**
- **No apparent rmIL-17E treatment related clinical signs of toxicity e.g. decreased body weight, morbidity, and abnormal changes in blood cell counts were observed at the end of the study.**

rm IL-17E Protein and IL-17E Receptor Expression Analysis in Mouse Cancer Cell Lines

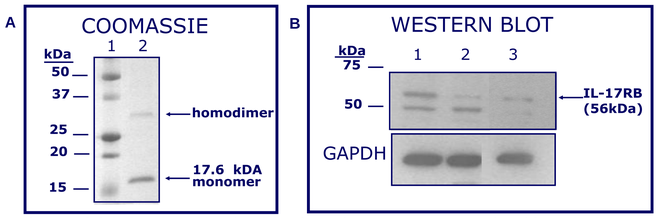


Figure 1. (A) SDS-PAGE: 12% gel analysis of recombinant mouse IL-17E purchased from R&D Systems, Minneapolis,MN. Lanes **(1)** Precision Plus protein standards (Biorad) **(2)** rmIL-17E: the 153 amino acid recombinant protein has a predicted molecular mass of 17.6 kDa (monomer) and the functional protein exists as a disulfide-linked homodimer. **(B) Western blot analysis of IL-17RB expression in mouse cancer cell lines: (1)** CT26 lysate and **(2)** B16-F10 lysate with **(3)** positive control human whole cell lysate from CML cells. IL-17RB(TJ-5): sc-73969 rat monoclonal antibody (500x) and goat anti-rat IgG-HRP: sc-2006 secondary antibody (10,000x) were used to detect of IL-17RB

In vitro Cell-Based IL-17E Sensitivity Assays

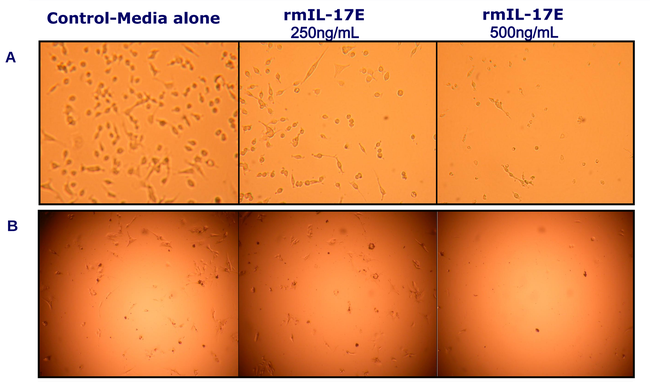


Figure 2. In vitro treatment with rmIL-17E of CT-26 and B16-F10 cells. 1 x 10^3 cells were seeded in 96-well microtiter plate in appropriate media and incubated for 24 hrs at 37^{0}C under 95% air and 5% CO_2. rmIL-17E at 250 ng/mL and 500 ng/mL in fresh media were added to approriate wells and incubated for 24 hrs under same condition. After 24 hr treatment with rmIL-17E, **(A)** CT26 and **(B)** B16-F10 cells were observed under the microscope. Decreased number of cells were observed compared to untreated control. **(C)** cell viability was quantitated using the XTT (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium}-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) colorimetric assay following manufacturers protocol (Roche Applied Science). Decreased number of cells at 500ng/mL was significant (*P<0.05).

Apoptosis Assay

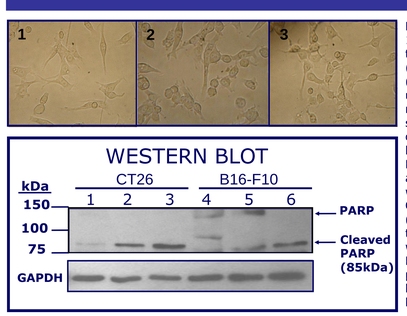


Figure 3. Apoptosis assay. (A) 2 x 10^5 CT26 cells were seeded in 6 well tissue culture plate and treated with **(1)** RPMI media alone, **(2)** rmIL-17E (10ug/mL) and **(3)** Staurosporine (1 uM) for 24 hrs. rmIL-17E induced rounding of cells similar to Stauro-sporine to a lesser degree within 4 hrs of treatment. **(B)** Respective cell lysates were prepared from CT26 and B16-F10 cells after 24 hrs of treatment as described above. 30 ug of proteins were loaded in 12% SDS-PAGE gel. Control (lanes1 and 4),rmIL-17E treated (lanes 2 and 5), and Staurosporine treated (lanes 3 and 6) were probed with anti-PARP antibody (51-6639GR,BD Pharmingen) following recommended protocol. Intact PARP is identified as a band of 116 kDA and cleaved PARP as band of 85 kDA.

In vivo Mouse CT26 Syngeneic Tumor Model

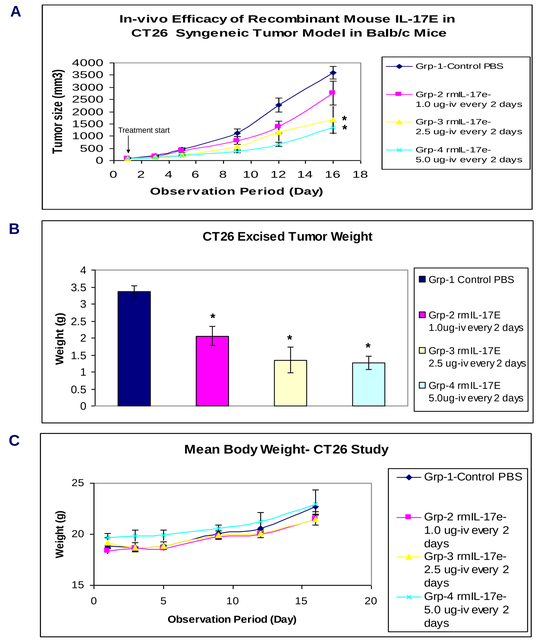


Figure 4. Anti-tumor activity of rmIL-17E against mouse CT26 colon tumors at different dose levels. A) Plot of sizes of subcutaneous tumors in Balb/c mice over the course of the study. Mice were inoculated with 1 x 10^6 CT26 cells and treatment started 7 days post-inoculation. Tumor sizes (mm^3) were measured at each point shown in the growth curve using the formula (V=L x W^2/2). Mice (n=5) were treated with rmIL-17E by i.v. injection at 1ug/mouse, 2.5ug/mouse, and 5.0ug/mouse every 2 days (For a 20g mouse, this is equivalent to 0.05 mg/kg, 0.125mg/kg, and 0.25 mg/kg dose levels, respectively). Control group was given 100uL PBS by i.v. injection every 2 days. All treatments were administered for the duration of the study. Significant anti-tumor efficacy was achieved at 2.5ug/mouse and 5.0ug/mouse (*P<0.05) dosing regimens in a dose-dependent manner. **B)** Excised CT26 tumor weight measurements at the end of the in-vivo efficacy study with rmIL-17E. CT26 tumors treated with rmIL-17E at 1.0ug, 2.5ug and 5.0ug showed a statistically significant reduction in tumor weights in a similar dose-dependent manner (* P<0.05). **C)** Mean body weight profile.

B16-F10 Lung Metastasis Model in Balb/c Mice

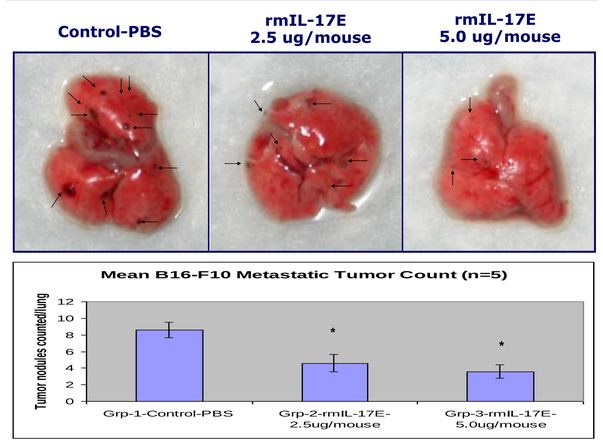
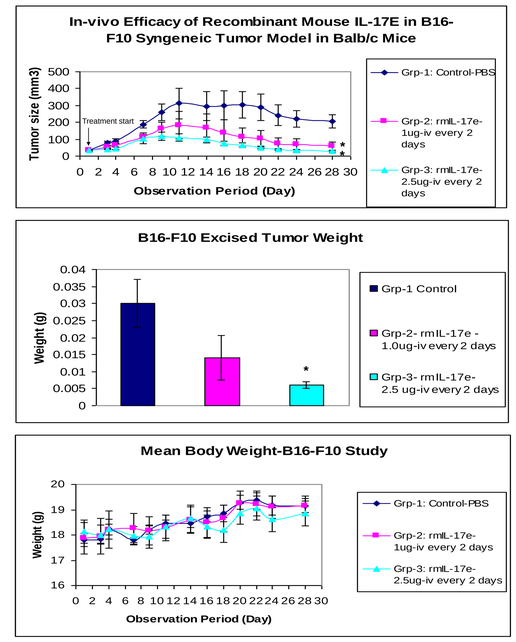


Figure 5. Anti-metastatic activity of rmIL-17E in lung tissues following B16-F10 melanoma cell inoculation. Mice (n=5) were inoculated with 1 x 10^4 B16-F10 cells in the tail veins. Treatment groups were pre-treated with rmIL-17E at 2.5 and 5.0 ug/mouse 24 hrs and 2 hrs prior to cell inoculation and treated every 2 days. **(A)** Excised lung tissues showing more visible tumor nodules / foci in the control group vs treatment groups 16 days post-cell inoculation (indicated by arrows). Control lungs have greater number and larger tumor nodules /foci compared to rmIL-17E treated lungs.**(B)** The reduced mean tumor counts in the rmIL-17E treated groups (2.5ug/mouse, and 5.0ug/ mouse every 2 days) were statistically significant compared to the control (* p<0.05) by Student T test.Higher dose of rmIL-17E appears to confer better protection.

In vivo Mouse B16-F10 Syngeneic Tumor Model



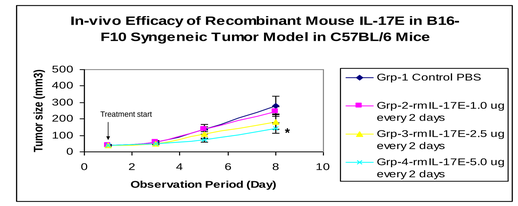


Figure 6. Anti-tumor activity of rmIL-17E against mouse B16-F10 melanoma tumors at different dose levels. A) Plot of sizes of subcutaneous tumors in Balb/c mice over the course of the study. Mice were inoculated with 2 x 10^6 B16-F10 cells and treatment started 3 days post-inoculation. Tumor sizes (mm^3) were measured at each point shown in the growth curve using the formula (V=L x W^2 /2). Mice (n=5) were treated with rmIL-17E by i.v. injection at 1ug/mouse and 2.5ug/mouse every 2 days (For a 20g mouse, this is equivalent to 0.05 mg/kg and 0.125mg/kg dose levels, respectively). Control group was given 100uL PBS by i.v. injection every 2 days. All treatments were administered for the duration of the study. Significant anti-tumor efficacy was achieved at 1.0ug/mouse and 2.5 ug/mouse (*P<0.05) dosing regimens in a dose-dependent manner. **B)** Excised B16-F10 tumor weight measurements at the end of the in-vivo efficacy study with rmIL-17E. B16-F10 tumors treated with rmIL-17E at 2.5 ug showed a statistically significant reduction in tumor weights (* P<0.05). **C)** Mean body weight profile. **D)** C57BL/6 mice (n=7) were inoculated with 7.5 x 10^5 B16-F10 cells and treatment started 3 days post-inoculation. rmIL-17E mediated a dose-dependent response with significant tumor growth inhibition achieved at the highest dose of 5.0 ug/mouse (*p=0.05).

Haematology Results

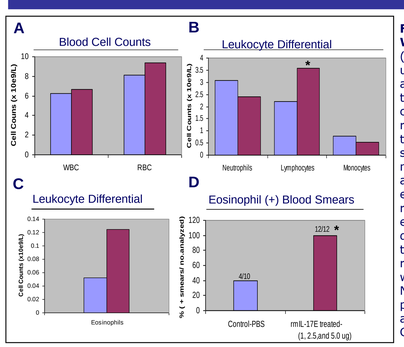


Figure 7. Complete blood cell and WBC differential counts. Balb/c mice (n=5) were treated with rmIL-17E (5.0 ug/mouse) x 9 doses and blood samples analyzed. -PBS and -rmIL-17E treated groups. **(A)** Total WBC and RBC counts were within normal reference range in both control and rmIL-17E treated groups. **(B)** Differential counts showed normal relative numbers of neutrophils, lymphocytes, monocytes and **(C)** eosinophils. Lymphocyte and eosinophil counts appeared higher in rmIL-17e treated samples and **(D)** eosinophils were more frequently detected in blood smears of rmIL-17E treated groups ($p<0.05$). Normal reference range for blood cell counts were based on Charles River and Nemzek, et.al. Inflamm. Res. 50(2001) p523-527. Blood samples were analyzed at Animal Health Laboratory, University of Guelph, Ontario, Canada.