Int. J. Cancer: **106,** 528–533 (2003)

Publication of the International Union Against Cancer

NATURAL KILLER CELL CYTOLYTIC ACTIVITY IS NECESSARY FOR *IN VIVO* ANTITUMOR ACTIVITY OF THE DIPEPTIDE L-GLUTAMYL-L-TRYPTOPHAN

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A dipeptide, L-glutamyl L-tryptophan (L-glu-L-trp), was identified in a screen for immunomodulators in the soluble fraction of the thymus. L-glu-L-trp inhibits tumor growth in mice without showing direct cellular toxicity in a variety of human tumor cell lines. L-glu-L-trp antitumor activity *in vivo* requires the presence of natural killer (NK) cells. Defective trafficking of cytoplasmic granules caused by the *Lyst* mutation also resulted in loss of antitumor activity of the dipeptide. The effect of L-glu-L-trp on tumor growth in mice with targeted gene mutations demonstrated the absolute requirement for perforin for antitumor activity. The requirement of 2 major modulators of NK cell activity, gamma interferon (IFNγ) and interleukin (IL)-12, were also tested. L-glu-L-trp had full antitumor activity in IFNγ knockout mice, but had significantly diminished activity in IL-12 knockout mice. These data show that L-glu-L-trp antitumor activity in mice is dependent on cytolytic cell activity of NK or NKT cells. L-glu-L-trp *in vivo* regulates NK cell function independent of IFNγ but partly dependent on IL-12.

Key words: *antineoplastic; natural killer cells; immune modulatory*

The innate immune system comprises the body's first line of defense and acts prior to the elicitation of specific immunity. The innate immune system recognizes "nonself" entities such as microbes, parasites and abnormal cells. Loss of expression of major histocompatibility cluster (MHC) class I molecules, as a consequence of viral infection or neoplastic transformation, flags cells for destruction by innate immunity effector cells.[1] Cellular components of the innate immune system are: the phagocytic macrophages and neutrophils; cells that release inflammatory mediators such as basophils, eosinophils and mast cells; and natural killer (NK) cells. NK cells induce apoptosis of target cells that lack MHC class I surface expression.[2] MHC class I molecules are the ligands for the NK cell inhibitory receptor that overrides signals initiated by the binding of NK activating receptors. In the absence of MHC class I expression on the targets, NK cells initiate calcium-dependent formation of perforin pores and release of cytolytic granule serine proteases such as granzyme B that activates the caspase cascade to effect apoptosis.[3] The cytotoxic T-cells (CTL), a component of the adaptive immune response, also cause target cell death by the formation of perforin pores and release of cytolytic enzymes. However, recognition of target cells by CTL is antigen specific and restricted by MHC class II molecules.[4] NKT cells comprise a third cell type that possesses perforin-dependent cytolytic activity. NKT cells express NK specific markers and a limited T-cell receptor repertoire (reviewed in Brutkiewicz and Sriram[5]). Thus, these cells may bridge the gap between the innate and adaptive immune responses.

NK, NKT and CTL proliferation and activation is regulated by several cytokines including IFNα /β, IL-1, -2, -12, -15, -18 and tumor necrosis factor (TNF) (reviewed in Biron *et al.*[6]). The activities of each cytokine differ. For example, IFNα/β enhances NK cell proliferation, cytotoxicity and trafficking mainly in response to viral infection. On the other hand, IL-12 is critical for NK activity against nonviral targets. Both IL-2 and -15 preferentially induce proliferation and maturation of NK cells but do not necessarily lead to increased activity. The activating cytokines, such as IL-12 and -18, in turn induce the expression by NK cells of IFNγ. IFNγ is active in modulating the innate immune response and in shaping the subsequent adaptive response.

NK cells infiltrate tumors and may protect against tumor spread.[7] Approaches to enhance the antitumor potential of NK cells and the innate immune system have entered the clinic over the last decade (reviewed in Salgaller and Lodge[8]). However, systemic administration of the cytokines that regulate NK cell proliferation and/or activation perturb complex regulatory pathways and serious side effects have been observed, including hypotension, cachexia, fever, chills, vomiting and diarrhea. There is an obvious need for more specific immune modulators that lack the wide-ranging side effects of the cytokines currently found in the clinic.

Short peptides from thymic extracts have been found to have immunostimulatory activity.[9] The synthetic equivalent of the thymic extract dipeptide, L-glu-L-trp, was found to be nontoxic and inhibited the growth of tumors in animal models.[9,10] In our study we show that the inhibition of tumor growth *in vivo* by L-glu-L-trp is due in large part to modulation of NK and/or NKT cell activity.

MATERIAL AND METHODS

Cell lines and reagents

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA). The following cell lines were obtained from colleagues at the University of Southern California: M21 human melanoma[11] from P. Brooks, human ovarian carcinoma line Hey from L. Dubeau and T1 fibroblasts from P. Jones. KS Y-1 was isolated in this laboratory from an AIDS-Kaposi's sarcoma lesion and has been described previously.[12] KS-SLK was isolated from a classic Kaposi's sarcoma patient.[13] Lewis lung carcinoma (LLC) and B16 melanoma were purchased from the American Type Culture Collection (Manassas, VA).

Pan NK antibody DX5 and NK1.1 neutralizing antibody were obtained from BD-Pharmingen (San Diego, CA). Rabbit anti-mouse asialo GM1 polyclonal antibody was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY).

Mouse strains

Balb/C *nu/nu* and C57BL/6 mice were obtained from Simonsen Laboratories (Gilroy, CA); SCID-beige and C57BL/6 *nu/nu* from

Abbreviations: CTL, cytotoxic T-lymphocytes; HBSS, Hank's balanced salt solution; HUVEC, human umbilical vein endothelial cells; IFN, interferon; IL, interleukin; L-glu-L-trp, L-glutamyl-L-tryptophan; MFIR, mean fluorescence intensity ratio; MHC, major histocompatibility cluster; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NK, natural killer lymphocytes; s.c., subcutaneous; s.e.m., standard error of the mean; TNF, tumor necrosis factor; TRAIL, tumor necrosis-related apoptosis-induced ligand.

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Received 22 March 2002; Revised 14 August 2002, 25 March 2003; Accepted 3 April 2003

DOI 10.1002/ijc.11253

Taconic (Germantown, NY); C57BL/6J-*Lyst*$^{bg\text{-}J}$/*Lyst*$^{bg\text{-}J}$, C57BL/6-*Pfp*$^{-/-}$, C57BL/6-*Ifng*$^{-/-}$ and C57BL/6-*Il12b*$^{-/-}$ from Jackson Laboratories (Bar Harbor, ME); and C3H/HCG mice from Charles River Laboratories (Portage, MI). All mice were maintained in pathogen-free conditions in accordance with the University of Southern California institutional guidelines governing the care of laboratory mice.

Cell proliferation assay

Cells were seeded at a density of 1.0×10^4 cells/well in 24-well plates in an appropriate medium (Clonetics, San Diego, CA). The cells were allowed to attach overnight and then treated with L-glu-L-trp (0.1–1000 mg/ml) on days 1 and 3. Cell viability count was determined on day 5 by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.5 mg/ml as described previously.[14]

Flow cytometry

Cells were isolated from the spleens of C57BL/6 mice harvested at the conclusion of a tumor growth experiment. Cells were washed 3 times in Hank's buffered saline solution (HBSS) and 1×10^6 cells were stained with the pan anti-NK cell marker DX5 for 30 min on ice and washed twice with HBSS. Stained cells were analyzed by an FACS calibur machine (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Mean fluorescence intensity ratio (MFIR) was obtained by multiplying the percent positive cells with the mean fluorescence intensity of the positive cells.

In vivo *tumor growth*

Tumor cells propagated *in vitro* were inoculated subcutaneously in the lower back of 5-week-old male mice. Treatment was initiated 4–10 days after implantation. In experiments with the C57BL/6 IFN-γ and IL-12b knockout mice, treatment was initiated the day after tumor implantation because tumor growth was very aggressive in these strains. L-glu-L-trp was tested at various concentrations ranging from 0.1–100 mg/kg/day given subcutaneously for an average of 2 weeks. The controls consisted of PBS, L-glutamic acid, L-tryptophan, or L-alanyl-L-alanine. In the NK cell ablation experiments, asialo GM1 antibody was given twice a week intraperitoneally in C57BL/6. Tumor volume was measured (width^2 × length × 0.52) 3 times a week, and tumor weight was taken at the conclusion of the experiments.

Lung metastasis assay

Balb/C nu/nu mice were inoculated with 5×10^5 B16 melanoma cells by tail vein injection. Mice were randomly placed into groups of 6 mice each and received one of the following treatments by subcutaneous (s.c.) injection: L-glu-L-trp 10 mg/kg/day; L-glu-L-trp 100 mg/kg/day; or equal volume (100 μl) PBS in the control group. Treatment was started the same day as tumor cell inoculation and continued for 13 days. The mice were killed and the lungs harvested. The number of tumor lesions on the surface of the lungs was determined with the aid of a dissecting microscope.

Statistical analysis

Significance of differences in means of the control and treated groups was determined by Student's *t*-test.

RESULTS

In vitro *activity of L-glu-L-trp*

The dipeptide, L-glu-L-trp, was identified in a screening of the soluble forms of thymus extract to enhance T-cell rosetting.[15] The synthetic sodium salt is the subject of this report (Fig. 1*a*). The effects of L-glu-L-trp on tumor cells and HUVEC were determined *in vitro*. In a 5-day growth assay L-glu-L-trp had no effect on the viability of either the tumor cell lines or HUVECs (Fig. 1*b*). Thus L-glu-L-trp has no cytotoxicity to the cell lines tested that were used in the subsequent *in vivo* experiments

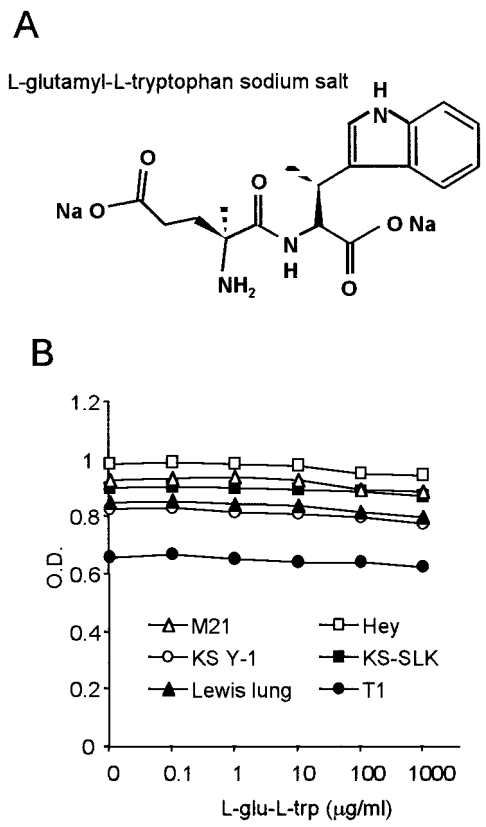

FIGURE 1 – Structure and *in vitro* activity of L-glu-L-trp. (*a*) L-glu-L-trp is the synthetic sodium salt of the dipeptide L-glutamyl-L-tryptophan. The chemical structure is shown. (*b*) *In vitro* activity of L-glu-L-trp. Cells (1.0×10^4 cells/well) were treated with L-glu-L-trp (0.1–1000 mg/ml) on days 1 and 3. Cell counts were performed on day 5 by MTT assay. Shown are the means ± standard deviation (SD)s of triplicate samples.

L-glu-L-trp has antitumor activity in vivo

The activity of L-glu-L-trp as an antitumor agent was investigated using murine tumor xenograft models. Figure 2 shows the results of experiments in both immune competent and immune deficient mice. LLC s.c. xenografts were implanted in C57BL/6 mice (Fig. 2*a* and *b*) and allowed to develop until the tumor was measurable at the subcutaneous site before s.c. administration of L-glu-L-trp (1–100 mg/kg/day) was begun. L-glu-L-trp resulted in a dose-dependent inhibition of LLC tumor growth in syngeneic immune competent mice (Fig. 2*a*). The antitumor activity of L-glu-L-trp is specific to this peptide since the same amount (100 mg/kg/day) of a control peptide, L-alanyl-L-alanine, had no significant effect ($p = 0.371$, compared to control) on tumor growth in the same model (Fig. 2*b*). In contrast, L-glu-L-trp resulted in a significant inhibition of tumor growth to 48% of control tumor volume ($p = 0.029$). L-glu-L-trp also has antitumor activity against established human tumors *in vivo* in immune deficient mice. L-glu-L-trp showed a dose-dependent decrease in tumor volumes of xenografts in Balb/C athymic mice implanted with either M21 human melanoma cells or Hey human ovarian carcinoma cells (Fig. 2*c* and *d*). L-glu-L-trp showed stronger antitumor activity towards M21 melanoma than to Hey ovarian carcinoma, which may either be attributable to intrinsic properties of the tumors,or to the tumor growth rate. The Hey tumor xenograft controls attained a size approximately 3-fold larger after 15 days than the M21 xenografts. Reduced tumor growth is not attributable to toxicities from L-glu-L-trp. No differences in nontumor body weight were seen after the animals were killed and tumors removed for histologic analysis. A preclinical acute toxicity trial

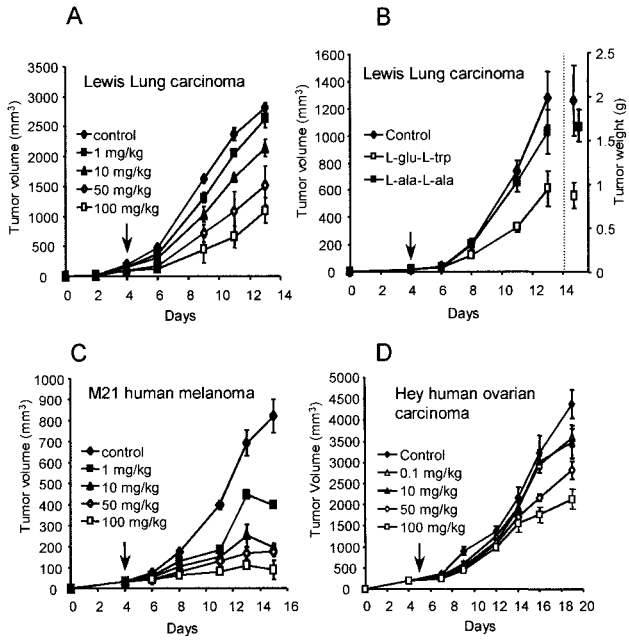

FIGURE 2 – L-glu-L-trp has antitumor activity in immunocompetent and immunodeficient mice. Tumor xenografts were initiated by s.c. inoculation of cell lines in the lower back of mice as described in Material and Methods. (*a*) Dose response to L-glu-L-trp in immunocompetent C57BL/6 mice. Lewis lung carcinoma xenografts were initiated with 2×10^6 cells and L-glu-L-trp was administered s.c. daily, beginning on day 4 (arrow) for the duration of our study. *p*-values by Student's *t*-test for treatment groups *vs*. control: 0.611 (1 mg/kg/day), 0.279 (10 mg/kg/day), 0.012 (50 mg/kg/day) and 0.002 (100 mg/kg/day). (*b*) Specificity of L-glu-L-trp antitumor activity. Lewis lung tumor xenografts in C57BL/6 mice as in (*a*), except that 7.5×10^5 cell were implanted. Mice received daily s.c. L-glu-L-trp or L-alanyl-L-alanine, both at 100 mg/kg starting on day 4. Final tumor weights are shown to the right of the growth curves. *p*-values for treatment groups *vs.* control: 0.371 (ala-ala), 0.029 (glu-trp). (*c,d*) Dose response of human tumor xenografts to L-glu-L-trp in Balb/C athymic mice, which lack mature T-cells. 3×10^6 M21 human melanoma cells (*c*) or 2.5×10^6 Hey human ovarian carcinoma cells (*d*) were injected subcutaneously to initiate tumor xenografts. Treatment with L-glu-L-trp was as in (*a*), starting at day 4 (M21) and day 5 (Hey). For all experiments the means ± standard error of the mean (s.e.m.) ($n = 6$) are shown.

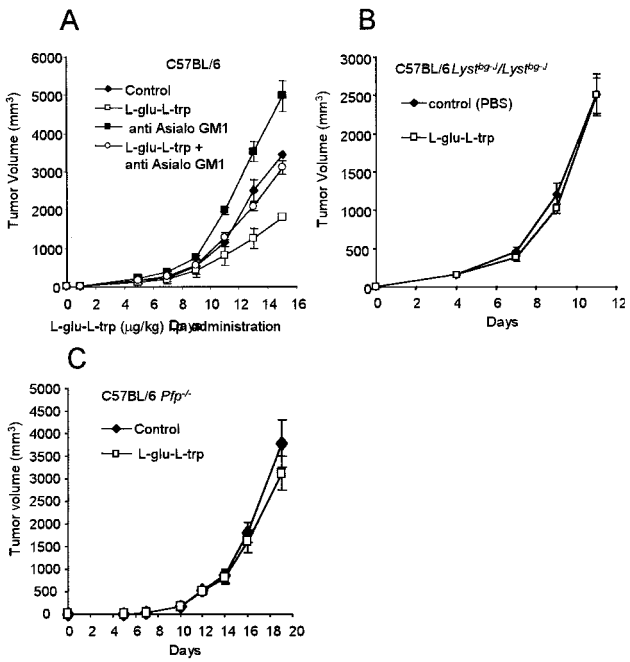

FIGURE 3 – NK cell involvement in L-glu-L-trp *in vivo* activity. (*a*) Cytotoxicity assay of splenocytes from C3H/HCG mice treated with L-glu-L-trp as indicated. Cytotoxic activity was determined by the standard chromium release method with YAC cells. A standard drug free vehicle control and negative Cyclophosphamide (50 μg/kg) control reference were used in testing NK cell activity. Cyclophosphamide showed the expected 25% decrease in NK activity (not shown). (*b*) *In vivo* NK cell ablation reduces the antitumor activity of L-glu-L-trp. Subcutaneous tumor xenografts of LLC tumors were initiated in C57BL/6 mice with 7.5×10^5 cells. Daily s.c. dosing with 100 mg/kg L-glu-L-trp was initiated in the treatment groups the following day. Anti asialo GM1 antibody (50 μl) was given i.p. on days –1, 3, 7 and 11. (*c*) L-glu-L-trp has no antitumor activity in C57BL/6 *Lyst*$^{-/-}$ (beige) mice in which cytolytic cells are nonfunctional. Subcutaneous tumor xenografts were initiated by implantation of 7.5×10^5 LLC tumor cells. Treatment with L-glu-L-trp (100 mg/kg/day) was initiated on day 3 of tumor growth. (*d*) L-glu-L-trp has no antitumor activity in perforin gene knockout mice. C57BL/6 *Pfp*$^{-/-}$ mice were inoculated subcutaneously with 7.5×10^5 LLC cells. Treatment with L-glu-L-trp (100 mg/kg/day) was initiated on day 4. For all *in vivo* experiments (*b–d*) the tumor growth curves shown represent the mean ± s.e.m. of 6 mice/group. In all cases control mice received an equal volume (100 μl) PBS s.c. daily. Results shown are representative of duplicate experiments.

conducted with a dose of 100 mg/kg/day for 15 days found no changes in the body weights of animals, food consumption values or visible abnormalities on postmortem examination.

Requirement of NK cell function

Since L-glu-L-trp antitumor activity is intact in athymic Balb/C mice, this indicates that T-cell function is not an absolute requirement for the dipeptide function. The *in vivo* effect of L-glu-L-trp on NK cells was then investigated. Flow cytometric analysis of splenocytes from tumor bearing immune deficient mice treated with L-glu-L-trp for 2 weeks indicated no change in NK cell numbers. The percent positive cells stained with antibody to the pan-NK marker DX5 was 6.4% in controls and was not appreciably different in any of the treatment groups, which ranged from 5.4–8.1%.

NK cells were depleted in C57BL/6 mice using anti-asialo GM1 antibody. LLC tumor xenografts were initiated by s.c. injection of 7.5×10^5 cells. On the following day L-glu-L-trp treatment (100 mg/kg/day) was initiated. Anti-asialo GM1 antibody was administered according to instructions provided by the supplier, which was 50 μl, i.p. the day prior to tumor implantation and every 4 days thereafter to the appropriate groups. The antitumor activity of L-glu-L-trp in NK cell depleted mice was markedly reduced (Fig. 3*a*). There was no significant difference by Student's *t*-test between tumors from untreated controls and mice receiving 100 mg/kg/day L-glu-L-trp together with anti-asialo GM1 antibody. In contrast, tumor sizes in L-glu-L-trp treated mice were significantly different from controls ($p = 0.003$) and showed a 50% reduction in mean tumor volume. There was also a significant difference in tumor volumes between L-glu-L-trp with anti-asialo GM1 and L-glu-L-trp alone ($p = 0.012$). Tumor growth in anti-asialo GM1 treated mice was faster than in the experimental controls, which is consistent with the removal of NK cell tumor surveillance.

Requirement for normal trafficking of cytoplasmic granules

The major mechanism of cytolytic immune cell mediated cell killing, including NK, NKT and CTL cells, is through release of cytolytic granule proteins into the target cells. The proper trafficking and exocytosis of the cytolytic granules is disrupted by a mutation of the *Lyst* gene in beige mice.[16] To test whether L-glu-L-trp antitumor activity depended on intact cytolytic machinery *in vivo,* the response of LLC xenograft growth in C57BL/6 *Lyst*$^{-/-}$

mice to L-glu-L-trp treatment was determined. LLC s.c. xenografts were allowed to establish for 2 days before starting L-glu-L-trp treatment (100 mg/kg/day). The rate of tumor growth in these mice was similar in both treated and untreated groups (Fig. 3*b*). No significant difference was seen between controls and treatment group ($p = 0.976$ for 100 mg/kg/day). In comparison, treatment of C57BL/6 wild-type mice bearing LLC tumors with the same dose schedule of L-glu-L-trp resulted in significant reduction in tumor volumes (Fig. 2*a* and *b*). Similarly, human KS Y-1 tumor xenografts in SCID-beige mice were not growth inhibited with 100 mg/kg/day L-glu-L-trp treatment (data not shown).

Perforin is required for L-glu-L-trp antitumor activity

NK, NKT and CTL cytolytic activity involves calcium-dependent release of granzymes and other proteolytic enzymes into the target cells via pores formed by perforin. Activation of these cells results in an increase in the number of cytolytic granules and clustering towards the contact region with the target cells. Loss of either perforin or granzymes results in failure of effector cell function.[17] We thus examined perforin deficient mice to further confirm the requirement of granular proteins in peptide activity. Subcutaneous LLC tumor xenografts implanted in C57BL/6 perforin gene knockout mice grew at the same rate in the 100 mg/kg/day L-glu-L-trp treated mice and controls (Fig. 3*c*). There was no significant difference in the size of the tumors in the 2 groups on day 19 ($p = 0.368$). This result established the critical requirement for perforin in L-glu-L-trp antitumor activity.

Contribution of IFNγ and IL-12 to L-glu-L-trp activity

We next wished to determine if L-glu-L-trp activity was dependent upon the known regulators of NK, NKT and CTL function and IFNγ and IL-12. LLC tumor xenografts were implanted in C57BL/6 IFNγ gene knockout mice to observe the antitumor effect of L-glu-L-trp in the absence of IFNγ. Figure 4*a* shows that 100 mg/kg/day L-glu-L-trp treatment resulted in 65% inhibition of the tumor volume, which was highly significant ($p = 0.009$). A lower dose of L-glu-L-trp (10 mg/kg/day) also resulted in a decrease in tumor volume, but the difference did not reach significance. Tumor weights measured at the end of the experiment were significantly different from controls for both 10 mg/kg/day and 100 mg/kg/day treatments ($p = 0.031$ and 0.009, respectively). These results indicate that L-glu-L-trp antitumor activity does not require IFNγ. In support of this, there was no evidence for induction of serum levels of IFNγ in mice treated on various studies (DLS, SZ, RM personal communication). In contrast, LLC tumor xenografts implanted into C57BL/6 IL-12 knockout mice showed a decreased inhibition of tumor growth at 100 mg/kg/day of 40% (Fig. 4*b*). The difference in this case between the controls and treated mice did not attain significance ($p = 0.070$). For the lower dose of 10 mg/kg/day of L-glu-L-trp a 24% inhibition of tumor growth was observed, which was not significant ($p = 0.157$). Tumor weights measured at the end of the experiment were not significantly different from controls for both 10 mg/kg/day and 100 mg/kg/day treatments ($p = 0.646$ and 0.157, respectively). This data suggests that IL-12 is necessary for the full effect of L-glu-L-trp.

Effect of L-glu-L-trp on metastasis

Perforin is required for NK cell protection against metastasis.[18] Having shown that perforin is required for L-glu-L-trp antitumor activity in a primary tumor model, we next investigated whether L-glu-L-trp could increase protection against metastasis. B16 cells were inoculated into the tail veins of mice. The number of lung metastases were quantitated after 13 days of treatment with 10 or 100 mg/kg/day L-glu-L-trp and compared to the number of lung lesions in mice that had received PBS (control group). A trend towards fewer B16 lung tumors was seen in mice treated with both 10 and 100 mg/kg/day L-glu-L-trp compared to controls (Fig. 5), however the data did not attain statistical significance (Student's *t*-test).

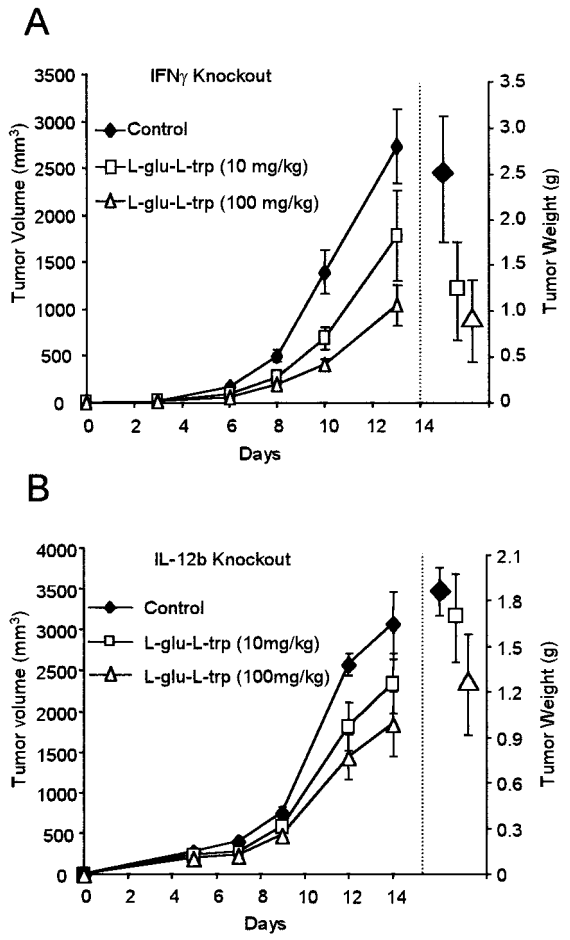

FIGURE 4 – Effect of L-glu-L-trp *in vivo* requires IL-12, but not IFNγ. C57BL/6 *Ifng*$^{-/-}$ (*a*) or C57BL/6 *IL-12b*$^{-/-}$ (*b*) mice were inoculated subcutaneously with 7.5×10^5 LLC. Treatment with L-glu-L-trp (100 or 10 mg/kg/day) was initiated the day after tumor cell implantation. Tumor growth curves and final tumor weights shown represent the mean ± s.e.m. of 6 mice/group. Control mice received an equal volume (100 μl) PBS s.c. daily. Results shown are representative of duplicate experiments.

DISCUSSION

Chronic treatment of outbred rats and C3H/sn mice with L-glu-L-trp for 12 months or longer has been shown to significantly decrease the incidence of spontaneous tumors to levels 65–50% of that seen in controls in both species.[9,10] Treatment of thymectomized mice with L-glu-L-trp resulted in a significant increase in activated T-cell differentiation and induced changes in intracellular cyclic nucleotides.[9] This supports an immunomodulatory role for the dipeptide in antitumor activity. We determined that L-glu-L-trp has antitumor activity *in vivo* against a variety of tumor cell xenografts, yet had no direct cytotoxic effects *in vitro* against the same cells. In our study, L-glu-L-trp was effective in inhibiting the growth of tumor xenografts in immunocompetent C57BL/6 mice and in athymic (BALB/c *nu/nu*) mice, which argues that the antitumor activity does not depend on modulation of T-cell functions. Control of tumor growth and spread by NK cells has long been known.[19] We therefore investigated whether L-glu-L-trp antitumor activity could be due to modulation of NK cell activity.

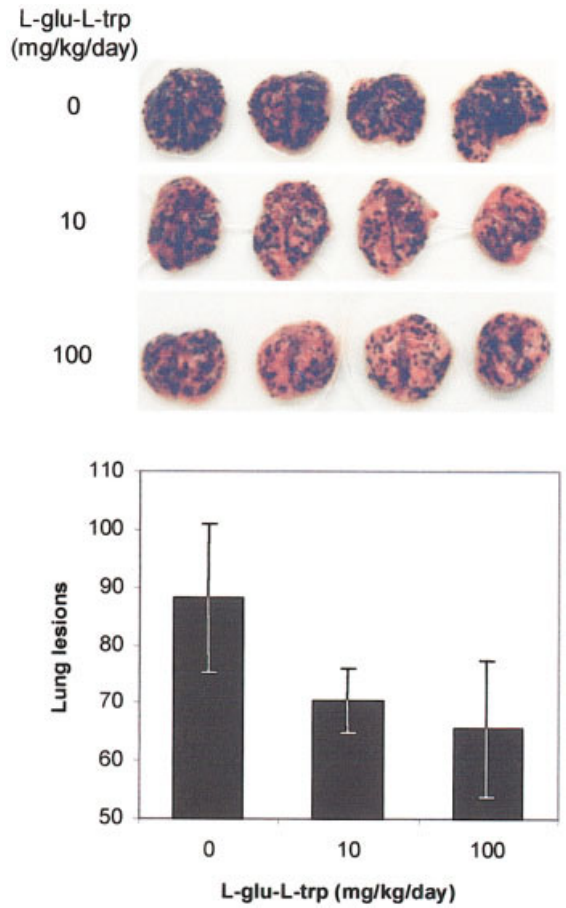

FIGURE 5 – L-glu-L-trp decreases the metastatic spread of tumors. Lungs of Balb/C nu/nu mice inoculated with 5.0×10^5 B16 melanoma cells treated with 0, 10 or 100 mg/kg/day L-glu-L-trp for 13 days. Black spots indicate tumor foci. Fewer tumors are seen in lungs from mice treated with L-glu-L-trp. The number of tumors/lung is shown in the lower panel.

NK cells from peptide treated mice were similar in number but the target cell killing was increased. The role of NK cell function was further confirmed in NK cell depleted mice using antiasialo GM1. Anti-asialo GM1 antibody effectively depletes the NK cell population in C57BL/6 mice.[20] The activity of L-glu-L-trp was significantly reduced suggesting that the dipeptide in part mediates its function through NK cells. It is also possible that NKT cells contribute to the antitumor effects of L-glu-L-trp since NKT cells are also depleted by anti-asialo GM1. The question of the involvement of NKT cells cannot be resolved by the use of C57BL/6 athymic mice, as there is strong evidence for the presence of and extrathymic maturation of NKT cells in these mice.[21]

We next investigated what functions of NK cells were necessary for L-glu-L-trp antitumor activity. NK mediated tumor control has been shown to proceed through induction of apoptosis in target cells and through either the TNF/TRAIL pathway[22] or the calcium-dependent release of cytolytic serine proteases from secretory granules.[3] We demonstrated that L-glu-L-trp antitumor activity required the granule exocytosis pathway by the use of C57BL/6 *Lyst*$^{-/-}$, SCID/beige and perforin knockout mice. The Lyst (beige) mutation in mice is homologous to a mutation in humans found in Chediak-Higashi syndrome, an hereditable disease that is associated with a defective immune system, increased risk of infections with bacteria, parasites and early death due to loss of function of NK cells and CTLs (reviewed in Introne *et al.*[24]). In both mice and humans, a characteristic of the disease is the presence of huge cytoplasmic granules in circulating granulocytes. A defect in a membrane fusion protein, the product of the *Lyst* gene, prevents lytic granule exocytosis.[24] Lack of L-glu-L-trp control of tumor growth in C57BL/6 *Lyst*$^{-/-}$ or SCID/beige mice established that exocytosis of granule proteins from cytolytic cells was necessary for *in vivo* antitumor activity. The complete lack of tumor growth control by L-glu-L-trp in perforin knockout mice confirms this result. Polymerization of perforin to form pores in target cells is required for entry of the granzymes that effect apoptosis.[3] Perforin knockout mice are functionally deficient in all the granzymes. Therefore we cannot rule out the possibility that one or more of this family of proteins is required for L-glu-L-trp antitumor activity *in vivo*.

Having determined that the NK and/or NKT cell granule exocytosis pathway is required for L-glu-L-trp antitumor activity, we next investigated the soluble factors, IFNγ and IL-12. IFNγ, a major product of activated NK cells, was not necessary for L-glu-L-trp antitumor activity. IFNγ is produced by NK cells in response to stimulation by a number of cytokines including IL-12, -18 and TNFα.[6] It is a pleiotropic cytokine with activities in innate immunity, inflammatory response and angiogenesis. Although NK cells express the high affinity receptor for IFNγ,[25] IFNγ does not appear to be involved in regulation of cytotoxicity of NK cells, as IFNγ production and cytotoxicity are distinct.[26] Our results showing full antitumor activity of the dipeptide in IFNγ knockout mice indicate that the TNF/TRAIL induced apoptotic pathway is not involved in this effect because IFNγ is required for the expression of TRAIL on NK cells.[22] We cannot rule out the impact of L-glu-L-trp on the TNF/TRAIL induced apoptosis of target cells from our study. However, TNF appears to be more important in the trafficking of NK cells and control of metastasis than in immediate regulation of granule release and target cell killing.[22,27]

IL-12 was originally isolated as an NK cell stimulating factor that induced expression of IFNγ.[28] Its major biologic effect was regarded as the stimulation of IFNγ production in NK cells and CTLs. However, recent reports indicate that it has activity independent of the stimulation of IFNγ. IL-12 can directly induce expression of perforin and granzyme B,[29] and the perforin pathway is the dominant apoptotic pathway in IL-12 antitumor effects.[30] In addition, NK cells stimulated with IL-12 and IL-18 do not require IFNγ for killer activity.[31] Our results, showing reduced antitumor activity of L-glu-L-trp in IL-12 knockout mice but full activity in IFNγ knockout mice, are consistent with the stimulation of cytolytic activity by IL-12 without the involvement of IFNγ. The fact that there was some antitumor activity in IL-12 knockout mice could indicate the participation of other factors in the dipeptide control of tumor growth. One such possibility is IL-18.

We have shown that the antitumor activity of L-glu-L-trp is mediated in part by cytolytic activity of perforin-expressing NK and/or NKT cells. Clearly, since L-glu-L-trp had no antitumor activity in both perforin gene targeted mutant mice and Lyst$^{-/-}$ mice, the antitumor mechanism is absolutely dependent on perforin exocytosis. We have shown that L-glu-L-trp regulation of NK and/or NKT cell cytotoxicity does not require IFNγ and is partially dependent on IL-12. Our data also supports a role for L-glu-L-trp in protection against tumor metastasis. There was a clear trend of fewer lung metastases observed. This is consistent with L-glu-L-trp modulation of NK cells that have a central role in the control of metastatic spread of tumors.[18] Whether the dipeptide directly regulates NK and/or NKT cell function through binding to a cell surface receptor or stimulates another cell type, such as monocytes or macrophages to produce cytokines that stimulate cytolytic activity in NK or NKT, is currently under investigation.

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