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■ RESEARCH ARTICLE

Generation of Safer Therapeutic Cytokines for Cancer Treatment

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ABSTRACT: A number of therapeutic monoclonal antibodies that target immune checkpoint proteins such as programmed cell death protein-1 (PD-1), and PD Ligand 1 (PDL-1) de-couple tumor's immune evasion mechanisms and have delivered significant treatment benefits to some cancer patients. However, 60 to 80% of cancer patients do not respond to these 'defensive-immunotherapies.' The limitations of defensive-immunotherapies may be effectively addressed by offensive-immunotherapy strategies. Therapeutic cytokines represent a class of offensive-immunotherapies and have demonstrated their potential in treating cancer patients but have not been widely utilized in clinics due to severe adverse side effects. In this article, development of safer-cytokine Interleukin 2 (IL-2) as "pro-cytokine" composed of IL-2 and its inhibitor domain fused via protease cleavable linker peptide is reported. The pro-cytokine IL-2 is designed to be activated by proteases at the tumor site while remaining in an 'predominantly inactive' state in circulation, thus eliminating the risk of systemic toxicity. The fusion gene construct was successfully designed and expressed in mammalian cells and IL-2 was released from the fusion protein when treated with matrix metalloprotease (MMP) and fully functional. The pro-cytokine IL-2 further demonstrated its therapeutic potential by remaining in an inactive state in serum.

KEYWORDS: Immunotherapy, Conditionally Activatable Cytokine, CTL (Cytotoxic T Cell), Pro-Cytokine, Matrix Metalloprotease, Defensive Immunotherapy, Offensive Immunotherapy, Cytokine, IL-2.

■ Introduction

Along with surgery, various forms of cancer treatment options such as chemotherapy, radiotherapy, targeted therapy, and combination therapy have incrementally enhanced cancer treatment response through the past several decades (Figure 1). The cancer drug evolution generated more specific tumor-targeting agents, however even with precise genetic profiling of tumors, targeted therapy was not sufficient to treat the disease due to heterogeneous nature of underlying genetic causes of cancer. The incremental progress in oncology, however, dramatically changed when drugs designed to block tumor's immune evasion mechanism thereby restoring patients' own immune system to fight cancer. The evolution of oncology drug development and clinical use mirrors our understanding of cellular disease development and systemic interaction of developing disease.

While the most effective method of treatment is surgery, this option is not beneficial for cancer patients with metastatic disease. Many decades ago, various forms of chemicals such as nitrogen mustard and various alkylating agents such as cyclophosphamide were used to treat various forms of cancer.[1] This led to the development of chemical agents interfering with the survival of rapidly replicating tumor cells. Among many chemical agents, folate antagonists such as methotrexate have demonstrated successful remission in children with leukemia.[2] 5-fluorouracil (5-FU) and its derivative molecules along with platinum-based agents are important chemotherapy options against many tumor types today. Higher treatment response rates were achieved by combining multiple chemotherapeutic agents targeting tumor cells in different phases of their cellular cycle with typical severe toxicities causing a significant decline in quality of life in patients.[3] However, chemotherapies are not a hundred percent effective and only improve progression-free or overall survival.

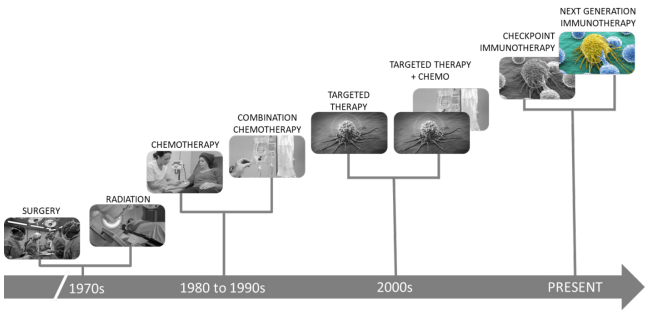

Figure 1: The evolution of cancer therapy over the past 50 years. With expanded understanding of tumor biology resulted in increase in therapy specificity substantially enhanced drug's ability to strategically target cancer cells selectively. With accumulated knowledge of systemic interaction between tumor cells and immune system, therapeutic approaches to restore anti-tumor immune function resulted in generating high rate of cure in many advanced stage cancer.

The development of modern computers enabled three-dimensional radiation-ray therapy, such as intensity-modulated radiation therapy (IMRT) using mapping information from Computed Tomography (CT) scans made tumor-targeting radiation therapy possible. The CT scans help minimize toxicity since the contours of the tumor are targeted and separated from healthy tissues. Molecular targeting radiotherapy takes advantage of tumor targeting proteins conjugated with

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radioactive isotopes (Iodine-125 or Indium-111) for potential delivery to specific tumor cells, thus sparing healthy cells.[4] However, radiation therapies rarely lead to complete disease-control.

Despite advancement of chemotherapy and radiation therapy, they do not provide long term clinical response and patients ultimately succumb to the deadly disease. However, when these therapeutic modalities are combined, enhancement of treatment response have been observed in some cancer types, therefore advanced stage cancer patients are now routinely treated by chemo-radiation combination therapy for incremental benefit of progression free survival.

Two major revolutions have changed cancer treatment paradigms in the past decade: targeting actionable alterations in oncogene-driven cancers and immuno-oncology. The combined accumulation of our understanding of cell biology and underlying genetic-drivers for cell proliferation allowed development of new classes of drugs categorized as 'targeted therapeutics.' They are either traditional small molecules selected through a screening process or monoclonal antibodies designed for their selective ability to inhibit key proteins that are responsible for cell proliferation. Most often these drugs target receptor proteins on cell surface Receptor Tyrosine Kinase (RTK) or cytoplasmic signal transduction pathway proteins to trigger pathways to instruct cells to express genes to promote cell proliferation or to inhibit programmed cell-death, apoptosis.[5] However, the treatable genomic alterations are diverse in nature due to multiple/redundant proliferation-signaling pathways. Therefore, it is critical to identify dysfunctional targetable tumor drivers and administer specific RTK inhibitors or pathway inhibitors to successfully control the disease. As these new class therapies are highly specific to their intended target protein(s), this requires selection of suitable cancer patients. Next-generation sequencing technologies are increasingly being implemented for molecular prescreening in clinics. However, it is challenging to deal with tumor heterogeneity and its acquired resistance.

Upon the recognition of tumor antigens presented by MHC-I molecules, T lymphocytes are activated to infiltrate into the tumor microenvironment (TME) to inhibit and kill tumor cells. However, tumor cells exploit numerous mechanisms to evade T cell-mediated killing. One such approach is to upregulate Immuno Checkpoints (ICs), a group of proteins that repress the function of immune cells including T cells. While ICs promote self-tolerance in normal physiological environments, in the context of cancer-physiology they allow immune evasion in malignant tissues. Therapeutic monoclonal antibodies targeting PD-1, PD-L1, or CTLA-4 have shown significant efficacy in treating a number of cancers. However, cancer patients often develop primary or acquired resistance to these drugs limiting their utility only in 20 to 30% of certain cancer patients.[6] As these Checkpoint Inhibitors require the patients' pre-existing anti-tumor immune cells to take advantage of decoupling immune evasive interactions, this approach leaves patients lacking anti-tumor immune cells resistant to this class of 'defensive immunotherapy' regimens.

The limitations of defensive-immunotherapies may be effectively addressed by offensive-immunotherapy strategies. Therapeutic cytokines represent a subclass of offensive-immunotherapies and have demonstrated their potential in treating end stage cancer patients but have not been widely used by oncologists due to the severity of their adverse side effects. For example, recombinant IL-2 is a potent T cell proliferation and differentiation factor and is an FDA approved immunotherapy for advanced melanoma and renal cell carcinoma. Despite the remarkable and durable remissions, however, the use of this drug has not been widely adapted by clinicians due to the severe systemic toxicity triggered by indiscriminate systemic activation of immune cells.[7] Consequently, the clinical use of therapeutic cytokines has been limited to well trained and approved intensive care centers for supervised administration of therapeutic IL-2. There is a critical unmet medical need to develop safer-cytokine therapy regimens. Efforts to develop safer therapeutic cytokines have led to diverse approaches to modify cytokines to reduce their systemic toxicity.

Efforts to make safer-cytokines IL-2 to treat cancer patients without systemic adverse effects by designing recombinant IL-2 with sub-inhibitor domain fused via short linker peptide led to the generation of safe 'pro-cytokine.' The pro-cytokine IL-2 may be given to a patient without adverse systemic toxicity as this drug may remain inactive within a patient. The peptide linker fusing IL-2 to inhibitor, however, is subject to the proteolytic process in an environment with high levels of proteases. Hyper protease activity is a hallmark of tumor-metastasis, hence pro-cytokines IL-2 can become active in tumor tissue and may trigger selective anti-tumor immune responses while remaining inactive in the body as its concept shown in Figure 2. This manuscript reports evaluation of pro-cytokine IL-2 recombinant proteins and their potential use in animal models which may lead to a drug design for potential use in treating cancers with minimal adverse effects.

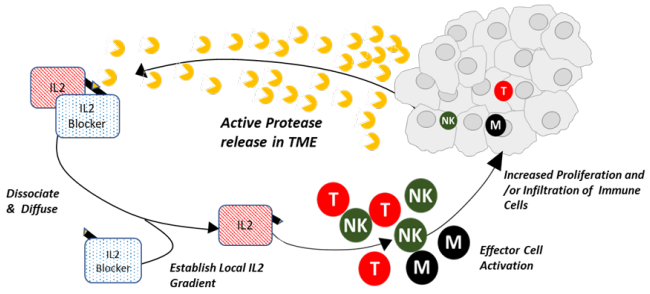

Figure 2: As pro-cytokine IL-2 is inactive as a pro-drug composed of cytokine IL-2 and its inhibitor module fused by linker-peptide, a substrate for matrix metalloprotease (MMPs). The pro-cytokine IL-2 remains inactive until cleaved by active-MMPs in tumor microenvironment. This selective TME-activating process ensures to trigger maximal anti-tumor immune response while minimizing systemic toxicity. Gray cells denote tumor cell cluster. NK= Natural Killer cells, M= Macrophage, T= T cells.

Goal: The primary goal of this study is to express pro-cytokine IL-2 proteins through transfecting mammalian cells and to evaluate by comparing fusion protein activity with or without protease treatment and to test its stability in serum obtained from tumor-bearing mice.

METHOD

Generation of Pro-cytokine IL-2 fusion proteins:

The gene regions encoding mouse IL-2 were genetically fused to IL-2 blocker peptide with bridging nucleotide sequences coding for MMP substrate peptide. The IL-2 blocker has adequate affinity for IL-2 where it can function as an IL-2 inhibitor while readily departing from the fusion protein when treated with MMP.[8] The c-terminal region of IL-2 blocker is fused to murine Fc gene to enhance its circulating time in the body and to be used for effective purification. The nucleotide sequences for the pro-cytokine IL-2 constructs were generated by gene synthesis (Genescript) and cloned into the mammalian expression vector pcDNA™3.1+ (Thermo Fisher) with flanking EcoRI and XbaI sites as outlined in the Figure 3. The pro-cytokine fusion gene constructs were used to transfect suspension-adapted Chinese Hamster Ovary (CHO) cells that have been adapted to high-density growing conditions using serum-free suspension culture medium (Thermo Fisher). Transfection reagents and enhancers were used per manufacturer's protocol to generate the highest yields possible in a transient expression system. The pro-cytokine IL-2 is designed to be non-functional without the separation of IL-2 blocker from the fusion protein. MMP treatment of pro-cytokine IL-2, however, releases fully functional active-IL-2.

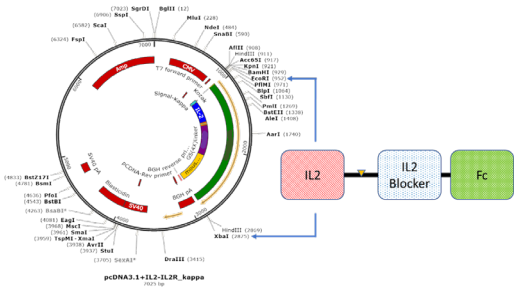

Figure 3: The schematic diagram of pro-cytokine IL-2-His and pro-cytokine IL-2-Fc are shown (left) with protease cleavable site within fusion-linker highlighted in yellow triangle. pcDNA3.1+ vector map with pro-cytokine IL-2 gene inserted via - EcoRI and XbaI sites is outlined (right). As there is no internal HindIII restriction site within fusion protein sequence, this site is engineered for future cloning strategy for cloning into GS vector for stable CHO cell line generation. pcDNA3.1+ vector map is from Thermo Fisher Scientific.

Generation of Pro-cytokine IL-2 fusion proteins:

Pro-cytokine IL-2 with *Fc*: HiTrap MabSelect SuRe (GE Healthcare) chromatography Protein-A column was used to purify secreted Fc tagged pro-cytokine IL12 from the ExpiCHO culture supernatants. Proteins bound to the resin were eluted with a low pH buffer or by competition with imidazole per manufacturer's protocol followed by size exclusion chromatography (HPLC SEC5 300A 7.8x300mm, 5µm part# 5190-2526, Agilent Bio). All purified samples were buffer-exchanged and concentrated by ultrafiltration to a typical concentration of > 1 mg/mL.

Cleavage of fusion protein by MMP9 protease:

Recombinant MMP9 (R&D Systems) was first activated with p-aminophenylmercuric acetate, and this activated protease or equivalent amounts of activating solution without the protease were used to digest or mock-digest the fusion protein overnight (18-22 hrs. at 37 °C). Cleavage assays were set up in in TCNB buffer: 50 mM Tris, 10 mM CaCl2, 150 mM NaCl, 0.05% Brij-35 (w/v), pH 7.5. The digested protein was aliquoted and stored at -80 °C prior to testing and aliquots of digests were analyzed by an SDS-PAGE followed by Western blotting to evaluate the extent of cleavage. Digests were also assessed in functional assays such as HEK-Blue Interleukin reporter assays.

Detection of expressed pro-cytokines:

Purity and homogeneity (typically > 90%) of final samples were assessed by an SDS-PAGE under reducing and non-reducing conditions. Purified proteins were aliquoted and stored at -80 °C until further use while untreated and digested fusion proteins were evaluated for cleavage products by Western blotting. Goat anti-mouse IL-2 polyclonal antibodies (AF-402-NA; R&D systems) were used to target IL-2 , and subsequent detection of immuno-complex was performed using a donkey anti-goat HRP-conjugated antibody (Jackson Immuno Research, West Grove, PA), and developed using the SuperSignal West Femto Maximum sensitivity detection reagent (Thermo Fisher) following the manufacturer's recommendations.

IL-2 functional cell-based assay:

IL-2 is a member of the four α helix bundle family of cytokines and shares the same signaling receptors with IL-2-Rβ and common γ chain receptors. Hence, activity of these cytokines was measured using the same reporter cell line HEK Blue IL-2 (Invivogen). HEK-Blue™ IL-2 cells were specifically designed to monitor the activation of the JAK-STAT5 pathway induced by ligand binding to the IL-2-Rβ and commonγ chain receptors. Stimulation with the appropriate cytokines triggered the JAK/STAT5 pathway and induced secreted embryonic xalkaline phosphatase (SEAP) production. SEAP was readily monitored using QUANTI-Blue™, a SEAP detection medium. For the HEK Blue assay, pro-cytokine IL-2 or activated-IL-2 were titrated and added to 50,000 HEK Blue cells per well in a 200 µL medium in a 96-well plate and incubated at 37 °C in 5% CO2 for 20–24 hours. The following day, levels of SEAP were measured by adding 20 µL of cell supernatant to QUANTIBlue reagent, followed by 1-3 hours of incubation at 37 °C and reading absorbance at 630 nm.

Serum Stability of Pro-Cytokine IL-2:

C57BL/6 mice were subcutaneously inoculated with MC38 murine colorectal cancer cells and when the average tumor volume reached 100 mm^3. Serum samples were collected from tumor bearing mice as well as control non-tumor bearing mice. Pro-cytokine IL-2 samples were spiked into a series of serum aliquots and incubated for up to 72 hours at 37°C. An ELISA assay was conducted to detect and quantify fusion proteins comprising IL-2 and IL-2 inhibitor moieties under each incubation condition. Wells of a 96-well plate were coated overnight with 100 µL of a rat anti-mouse IL-2 monoclonal antibody (JES6-1A12; Thermo Fisher) at 1 mg/mL in PBS. After washing, wells were blocked with TBS/0.05% Tween 20/1% BSA, then fusion protein samples were added for 1 hour at room temperature. After washing, an anti-mouse CD25 biotin-labelled detection antibody (BAF2438, R&D systems) was added, and binding was detected using Ultra Streptavidin HRP (Thermo Fisher). The ELISA plate was developed

by adding the chromogenic tetramethylbenzidine substrate (Ultra TMB, Thermo Fisher). The reaction was stopped by the addition of 0.5 M H2SO4, and the absorbance was read at 450-650 nm.

Results and Discussion

Generation of Pro-cytokine IL-2 fusion protein:

The fusion protein expression optimization process was required to maximize the yield by varying incubating duration of CHO cells. Recombinant proteins collected in extracellular media were purified through an affinity chromatography process as outlined in the method section. All purified recombinant proteins were buffer-exchanged and concentrated by ultrafiltration to a typical concentration of > 1 mg/mL. Purity and homogeneity (typically > 90%) of final samples were assessed by an SDS-PAGE under reducing and non-reducing conditions. Purified proteins were aliquoted and stored at -80 °C until further use. Purified pro-cytokine IL-2-Fc fusion proteins by Coomassie stain showed high purity of the target proteins and minimal high molecular weight entities. In the non-reducing condition, pro-cytokine IL-2-Fc fusion proteins are in a dimerized state mediated through Fc domain interaction like immunoglobulin. When reduced, Fc mediated dimer pro-cytokine IL-2-Fc displayed monomeric configuration as shown in Figure 4.

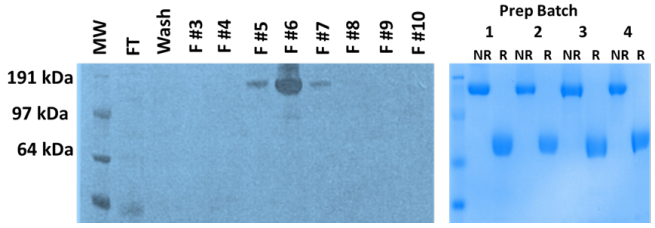

Figure 4: Affinity column chromatography (Ni-column for His tagged protein and Protein A column for Fc tagged fusion protein) mediated purified pro-cytokine IL-2 were analyzed by SDS-PAGE. Elusion fractions of protein A column retained Pro-Cytokine IL-2-Fc were collected in equal volume over 12 collection tubes. Flow through buffer and wash buffer did not contain the target recombinant protein. Elution buffer Fraction #5 to 7 contained pro-cytokine IL-2-Fc (left). Four (4) different transfection batches yielded robust Pro-cytokine IL-2-Fc (right) which displayed Fc mediated dimer configuration in non-reduced condition (lane NR) but reduced to approximately 70kDa monomer configuration (lane R). MW= Molecular Weight (kDa) ladder, FT= buffer Flow Through.

Detection, Cleavage of fusion protein by MMP9 protease:

Purified recombinant pro-cytokine IL-2 proteins were digested by chemically activated recombinant MMP9 proteins as described in the method section. The digested proteins were analyzed by an SDS-PAGE followed by Western blotting to evaluate the extent of cleavage. The recombinant pro-cytokine IL-2-Fc were incubated for 1, 4, or 20 hours with or without activated recombinant MMP9. Near complete cleavage of pro-cytokine IL-2-Fc was shown after 20-hour MMP treatment while no apparent cleavage was observed without functional MMP9 as shown in Figure 5. In order to test if proteolytically release IL-2 is functional a functional evaluation on reporter cell lines were conducted.

IL-2 functional cell-based assay::

HEK-Blue cells specifically engineered to monitor the activation of the JAK-STAT5 pathway induced by IL-2 binding to the IL-2-Rβγ chain receptors were utilized to quantitate IL-2-triggered JAK/STAT5 pathway as outlined in the Figure 6. HEK-Blue reporter cells displayed a substantially enhanced JAK/STAT5 pathway when pro-cytokine IL-2 was released by MMP9 treatment from the fusion protein. It required over 100-fold greater concentration of intact pro-cytokine IL-2 fusion protein to generate a similar reporter pathway activity level in the reporter cells. Baseline IL-2 activity in non-cleaved fusion protein is attributed to minor fusion protein with open configuration between cytokine and inhibitor module. With this proof of concept *in vitro*, it was very critical to demonstrate that pro-cytokine IL-2 fusion proteins may stay "inactive" without getting cleaved by circulating proteases.

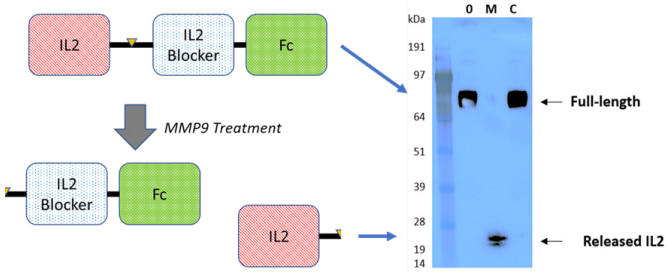

Figure 5: Pro-cytokine IL-2-Fc were treated with activated MMP9 for 16hr at 37°C. Resulting cleaved fusion proteins along with negative controls (fusion protein in buffer without MMP9) were separated on SDS-PAGE. Western blot analysis with anti-IL-2 antibody revealed increased amounts of released IL-2 from the fusion protein with longer incubation. Negative controls did not lead to IL-2 release from the fusion protein. 0= No Starting recombinant protein, M= Treated with MMP9, C= Control buffer treatment with no MMP9.

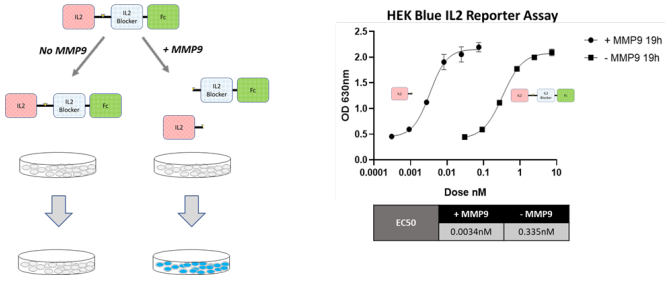

Figure 6: Schematic outline of functional analysis of IL-2 payload in pro-cytokine fusion protein with or without MMP9 treatment. Their ability to stimulate HEK Blue IL-2 reporter cells were tested by quantitating reporter-pathway activation. Two magnitude higher reporter-stimulation has been observed with proteolytic release of IL-2 from the fusion protein.

Serum Stability of Pro-Cytokine IL-2:

As anticipated, circulating proteases in serum regardless of the presence of tumor remain inactive as spiked pro-cytokine IL-2 remains stable over 72 hours as shown in the Figure 7. After incubating with serum for 4hr, 8hr, 24hr, 48hr, and 72hrs, the levels of spiked pro-cytokine IL-2 were detected using ELISA assay. The diverse types of matrix metalloproteinases are expressed as inactive enzymes requiring an enzymatic activation process within the tissue by other enzymes to separate the inhibiting domain from zymogens. As fully active MMPs may harm normal tissues, they are expressed as a zymogen (an inactive form of an enzyme) and require activation steps in the tissue. In addition to being expressed as an inactive form, there are tissue inhibitors of MMPs (TIMPs), in normal tissue to

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add additional mechanisms to maintain expressed MMPs in an inactive state. The successful demonstration of serum stability in tumor bearing mice assures that when injected into mice, pro-cytokine IL-2 will remain inactive thereby dramatically reducing systemic toxicity associated with fully active IL-2.

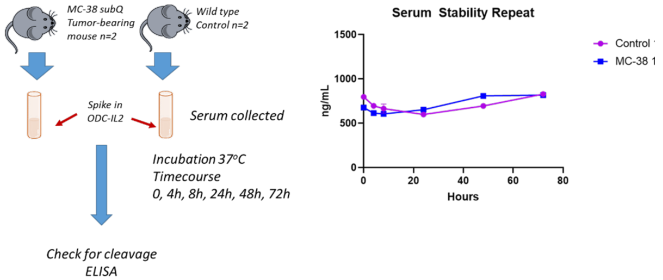

Figure 7: In order to see feasibility of pro-cytokine IL-2 for subsequence in vivo animal studies, fusion proteins were evaluated for their stability against proteases found in circulation. When mixed with serum collected from normal mice or tumor-bearing mice, fusion proteins remain intact up to 72 hours indicating circulating proteases are at inhibited state.

Conclusion

The series of experiments described and discussed here successfully demonstrated that therapeutic recombinant protein composed of cytokine may be expressed as inactive 'pro-cytokine' by integrating inhibitor domain fused but separable under unique hyperactive proteolytic conditions often found in advanced cancer disease stage.

Next Step: Conduct animal studies to demonstrate safety and determine tolerable dose of pro-cytokine IL-2 and subsequently to monitor its ability to inhibit tumor growth.

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