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NOVEL FUSION PROTEIN IL-12/FAS_{TI} FOR CANCER IMMUNOGENE THERAPY

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Department of Biological Sciences

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by
Xi Yang
May 2018

Accepted by
Dr. Yanzhang Wei, committee chair
Dr. Charlie Rice
Dr. Jeremy Tzeng
Dr. Xianzhong Yu

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ABSTRACT

Cancer immunotherapies using cytokines demonstrated effective in previous studies, whereas one major obstacle with the strategy is the severe side effects when administrated systemically at high doses. Additionally, cancer cells' escape from immune destruction has brought another challenge for cytokine therapy. Current approaches to cancer immunotherapy largely involve the immune factors designed to revive endogenous immune responsiveness, which renders high-efficient gene delivery system in urgent demand. In the first phase of our study, we demonstrated that fusion protein mIL-12/Fas_{TI}, encoded by cDNA of mouse interleukin-12 and transmembrane and intracellular domains of Fas, showed enhanced NK cell activity and anti-tumor cytotoxicity *in vitro* when expressed by stable tumor cell clones. As the second stage of study, a lentiviral vector-based gene delivery was used to deliver the fusion gene construct directly to tumor cells. Lentiviral vectors (pLenti7.3/IL-12/Fas_{TI} or Lent-IF) were constructed and used to transduce tumor cells. Preliminary data indicates that the constructs were efficiently delivered to tumor cells as assayed by RT-PCR and immunohistochemistry (IHC). The biological functions of the constructs as delivered by lentiviral system, such as NK cell activation, caspase-3 activity, Annexin-V apoptosis detection and tumor growth *in vitro,* have been demonstrated with statistical significance. This study represents a further step of bifunctional fusion protein cancer immunotherapy towards patient treatment.

Key words: Lentivirus, IL-12, Fas, NK cell, apoptosis, cancer immunotherapy

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DEDICATION

I dedicate this work to my mother Mingfen Yu and my father Jiabao Yang, who were longtime admirers of the beauty of the natural world. My family supported my interest in science and nature at a young age, and continues to do so today.

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CHAPTER ONE

LITERATURE REVIEW

The Biology of Cancer

Cancer has a major impact on society in the United States and across the world. Most recent statistics indicate that in 2016, an estimated 1,685,210 new cases of cancer will be diagnosed in the United States and 595,690 people will die from the disease. Up to now there are more than 100 different cancer types that have been identified, named specifically for the organ or type of cell in which they start.

The past two decades have witnessed tremendous advances in our understanding of the biology of cancer. It is now more clearly understood that cancer arises through a multi-step, mutagenic process with a plethora of underlying genetic causes in order to accomplish a proliferative advantage. In the course of remarkable progress in cancer research, it has been proposed that six hallmarks of cancer together constitute an organizing principle that provides a logical framework for understanding the remarkable diversity of neoplastic diseases (Hanahan and Weinberg, 2000). These include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Many of these phenotypic traits can be attributed to genomic instability that involves the gain-of-function mutation, amplification, and/or overexpression of key oncogenes together with the loss-of-function mutation, deletion, and/or epigenetic silencing of key

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tumor suppressors. Conceptual progress in the last decade has added two more emerging hallmarks of potential generality to this list—reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg, 2011). Importantly, we are beginning to see beyond the genes through the appreciation of significant role of epigenetic landscapes in the development of cancer (Flavahan, Gaskell and Bernstein, 2017).

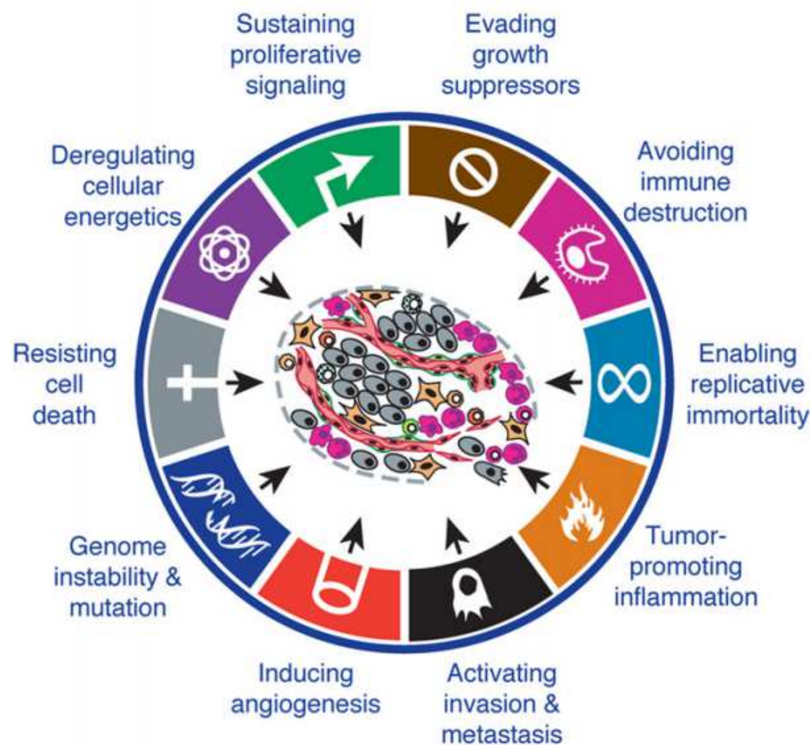

Figure1. Hallmarks of Cancer: the next generation (Hanahan D and Weinberg RD.2011)

Cancer evolves through random mutations and epigenetic plasticity that alter above discussed signaling pathways followed by clonal selection of cells that can survive and proliferate under circumstances that would normally be deleterious. Although a

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number of oncogenes and tumor suppressor genes, such as *p53* and *Rb*, are frequently mutated in cancer cells, there also appears to be a large number of low-frequency variances that contribute to oncogenic progress. Indeed, data from tumor sequencing projects reveal an astounding diversity of mutations in tumors. In one study, Stratton and colleagues estimate that individual mutations in as many as 20% of all kinases can play an active role in tumorigenesis (Greenman *et al.*, 2007). Therefore, better understanding of the biology of cancer disease and improvements in treatment are greatly needed. Increasing evidence supports the concept that a rare and specialized population of cancer cells, so-called cancer stem cells (CSCs) (or cancer-initiating cells) with stem cell-like characteristics, is responsible for tumor growth, maintenance, and recurrence. CSCs also exhibit characteristics that render them resistant to both radiation and chemotherapy, and therefore they are believed to take responsibility for treatment failure. This has led to the hypothesis that traditional therapies that indiscriminately kill tumor cells will not be as effective as therapies that selectively target cancer-initiating cells (Meacham and Morrison, 2013). Unraveling the underlying mechanisms of CSC's resistance to cancer treatments have contributed to novel therapeutic targets such as the discovery of CSC specific surface markers.

Furthermore, cancer is also an inflammatory disease. The relationships and mechanisms through which infection and inflammation promote tumor development were recently reviewed (Karin and Greten, 2005; Grivennikov, Greten and Karin, 2010). It is well established that chronic infections with hepatitis B and C viruses (HBV, HCV) represent major risk factors for development of hepatocellular carcinoma (HCC), while

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chronic H. pylori infection is the major risk factor for gastric cancer (Kuper, Adami and Trichopoulos, 2000). It was proposed that in both cases and others that the infectious agent leads to activation of NF-κB in myeloid cells (and eventually in lymphoid cells), resulting in production of growth and survival factors that stimulate tumor proliferation and development (Karin and Greten, 2005).

Cancer immune surveillance is considered to be an important host protection process to inhibit carcinogenesis and to maintain cellular homeostasis. In the interaction of host and tumor cells, three essential phases have been proposed: elimination, equilibrium and escape (Dunn, Old and Schreiber, 2004). During elimination stage, malignant cells can be eliminated initially and efficiently by immune effector cells such as NK cells and by the secreted IFN-γ in an innate immune response. The process of elimination includes innate and adaptive immune responses to tumor cells. Several effector cells such as NK, NKT and γδT cells are activated by the inflammatory cytokines, which are released by macrophages and stromal cells surrounding the tumor cells. The secreted cytokines recruit more immune cells, which produce other pro-inflammatory cytokines such as IL-12 and IFN-γ. Fas ligand (FasL) and TRIAL-mediated apoptosis of tumor cells by NK cells release tumor antigens which lead to adaptive immune responses (Zamai *et al.*, 1998; Smyth *et al.*, 2005). In the crosstalk between NK cells and Dendritic cells (DCs), NK cells promote the maturation of DCs and their migration to draining lymph nodes, resulting in the enhancement of antigen presentation to naïve T cells for clonal expansion of cytotoxic T cells (CTLs). The tumor antigen-specific T lymphocytes are recruited to the primary tumor site and directly attack and kill tumor cells with the

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production of cytotoxic IFN-γ (Zitvogel *et al.*, 2006). Elimination of transformed cells results in immune selection, which induces tumor variants that decrease immunogenicity and become resistant to immune effector cells in the equilibrium phase. These cells are more capable of surviving in an immunocompetent host. Eventually, when the tumor size can be detected by imaging diagnosis, tumor-derived soluble factors can induce several mechanisms for escape from immune attack in the tumor microenvironment(Dunn *et al.*, 2002). A tumor suppressive microenvironment can be established with activation and recruitment of myeloid-derived suppressive cells (MDSC), regulatory T cells and immune-inhibitory factors such as TGF-β and IL-10.

Any potential therapy to treat and possibly cure cancer must show differential toxicity toward tumor cells relative to normal cells. In principle, cancer can be treated by inducing cancer cells to undergo apoptosis, necrosis, senescence, or differentiation. These changes can be brought about by disrupting cancer cell autonomous processes, by interfering with autocrine/paracrine signaling within tumors, and/or by blocking heterotypic signaling between tumor cells and the surrounding stromal tissue or blood vessels. Enhancing immune surveillance against cancer cells expressing novel antigens is also an attractive approach that has shown efficacy in specifically killing cancer (Childs and Carlsten, 2015). The choice of cancer therapy depends upon the location and grade of the tumor and the stage of the disease, as well as the general state of the patient. This literature review will focus on the comparison of conventional and current strategies against different cancer types.

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Cancer Treatments

Surgery is one of the most commonly performed traditional cancer treatments. Theoretically non-hematological cancers can be cured if entirely removed by surgery in the absence of tumor metastasis. When the cancer has metastasized to other sites in the body prior to surgery, complete surgical excision is usually impossible. In addition to removal of the primary tumor, surgery is often necessary for staging, e.g. determining the extent of the disease and whether it has metastasized to regional lymph nodes. Staging is a major determinant of prognosis and of the need for adjuvant therapy.

Chemotherapy is the treatment of cancer with one or more cytotoxic anti-neoplastic drugs (chemotherapeutic agents) as part of a standardized regimen. Traditional chemotherapeutic agents act by killing cells that divide rapidly, one of the main properties of most cancer cells; hence, chemotherapy has the potential to harm normal tissues, especially those tissues that have a high replacement rate such as those in bone marrow, digestive tract and blood cells. Targeted therapy is a form of chemotherapy that can target specific molecular differences between cancer and normal cells, which act by blocking essential biochemical pathways or mutant proteins that are required for tumor cell growth and survival (Deininger and Druker, 2003; Druker, 2003). The first targeted therapy to be developed blocked the estrogen receptor molecule, inhibiting the growth of breast cancer (Long *et al.*, 2008). Another common example is the class of Bcr-Abl inhibitors, which are used to treat chronic myelogenous leukemia (CML). Currently, there are targeted therapies for breast cancer, multiple myeloma, lymphoma, prostate

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cancer, melanoma and other cancers. Dosage of chemotherapy can be difficult; if the dose is too low, it will be ineffective against the tumor, whereas, at excessive doses the toxicity (side-effects) will be intolerable to the patient (Corrie, 2011). In combination with surgery, chemotherapy has proven useful in a variety of different cancer types including breast cancer, colorectal cancer, pancreatic cancer, and certain lung cancers. The overall effectiveness of chemotherapy varies from being curative for some cancers such as leukemia (Nastoupil and Flowers, 2012; Nastoupil *et al.*, 2012), to being ineffective such as in some brain tumors because the blood–brain barrier poses a difficult obstacle to pass to deliver chemotherapy to the brain (Rampling, James and Papanastassiou, 2004). The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body.

Radiation therapy involves the use of ionizing radiation in an attempt to either cure or relieve the symptoms of cancer patients. The principle of radiation therapy is to damage the DNA of cancerous tissue leading to cellular death. To spare normal tissues such as skin or organs, which radiation must pass through to eliminate the tumor, shaped radiation beams are aimed from several angles of exposure to intersect at targeted tumor, providing a much larger absorbed dose than in the surrounding healthy tissues. The radiation is most commonly low energy X-ray beams for treating skin cancers, while higher energy X-ray beams are majorly used in the treatment within the body (Baskar *et al.*, 2012). As with chemotherapy, different types of cancers respond differently to radiation therapy.

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One major issue of these conventional cancer treatments including the combination of surgery and chemo-/radiation therapies is their toxicity, especially when tumor metastasis occurs. Furthermore, blood vessels in tumors, which are formed due to tumor angiogenesis, are very different from those in normal tissues. As a tumor metastasizes, tumor cells furthest away from the blood vessels become hypoxia. To counteract hypoxia, new blood vessels are signaled to grow. Because the newly formed tumor vasculature is poorly formed and does not deliver an adequate blood supply to all areas of the tumor, this leads to issues with drug delivery by the circulatory system (Minchinton and Tannock, 2006; Saggar *et al.*, 2013). Resistance is another major cause of treatment failure in chemotherapeutic drugs. There are a few possible causes of drug resistance in cancer, and one of primary reasons is anti-tumor drug efflux caused by ATP-driven pumps (Chen, Huang and Chen, 2013). It was demonstrated that cancer stem cells (CSCs) have an overexpression of ATP-binding cassette (ABC) transporters, which transport a wide variety of substrates including chemo-drugs from inside the cell to the outside.

The past few decades have seen a groundswell of research on the immune system yielding a deeper understanding of how cancer progresses and offering new therapeutic approaches to stop it. This progression has made the paradigm for cancer treatment to evolve from relatively non-specific cytotoxic agents to selective, mechanism-based therapeutics.

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Cancer immunotherapy is the use of immune system or its products to treat cancer. This strategy exploits the fact that cancer cells often have subtly different surface molecules that can be detected by the immune system. These molecules, known as tumor antigens, are most commonly proteins but also include molecules such as carbohydrates. Immunotherapies fall into several major groups: cellular, antibody, cytokine and/or combination of above. ***Cellular therapies***, also known as cancer vaccines, usually involve the removal of certain cancer cells or immune cells from cancer patients, modification with adjuvant materials to make complexes, and injection of the complexes back to patients. The purpose of cancer vaccines is to elicit a more powerful active immunity in the patient since any response of the patient's own immune system to tumor cells' immunosurveillance has clearly failed in cancer patients. Cancer vaccines include whole tumor cell vaccines, tumor-extracted protein vaccines, tumor antigen vaccines, dendritic cell (DC)-mediated vaccines and tumor antigen-encoding virus vaccines. These vaccines can be delivered alone or with adjuvants (Jager, Jager and Knuth, 2002; Palucka, Ueno and Banchereau, 2011; De Vries and Figdor, 2016). Cell types that can be used in this way are natural killer cells (NK cells), lymphokine-activated killer cells, cytotoxic T cells and dendritic cells (DCs). For instance, vaccination using dendritic/tumor cell hybrids (dendritoma) represents a novel and promising cancer immunotherapy(Wei *et al.*, 2006). DCs are professional antigen-presenting cells (APCs) and have been studied as an anti-tumor immune response activator in many ways, including tumor antigen pulsing, tumor antigen gene transfection and DC/tumor cell fusion (Rosenblatt, Kufe and Avigan, 2005; Palucka and Banchereau, 2013). This pilot study demonstrated that dendritoma vaccines

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could be administered safely to patients with metastatic renal cell carcinoma, while producing both clinical and immunologic evidence of response. Production and use of vaccines in this protocol were approved and monitored by the Food and Drug Administration (IND 9519)

Antibody therapies are the most successful immunotherapy, with approved treatments for a wide range of cancers. Antibodies are proteins produced by the immune system that bind to a target antigen on the cell surface. Antibodies are a key component of the adaptive immune response, playing a central role in both the recognition of foreign antigens and the stimulation of an antigen-specific immune response to them. It is not surprising, therefore, that many immunotherapeutic approaches involve the use of antibodies. The advantage of monoclonal antibody technology has made it possible to raise antibodies against specific antigens, such as the unusual antigens present on tumor surfaces. Monoclonal antibody therapy uses monoclonal antibodies (mAb) to specifically bind to specific cells or proteins against malignance, so as to stimulate the patient's immune system to attack cancer cells. Antibodies are also referred to as murine, chimeric, humanized and human. Murine antibodies were the first to be produced, which carry a great risk of immune reaction, because the antibodies are from a different species. Chimeric antibodies were the first attempt to reduce the immunogenicity of these antibodies. Chimeric antibodies are murine antibodies with a specific part of the antibody replaced with the corresponding human constant region. Humanized antibodies are almost completely human; only the complementarity determining regions of the variable regions are derived from murine antibodies. Human antibodies have complete human

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DNA (Harding *et al.*, 2010). In the past decade, patients with difficult-to-treat cancers such as advanced stage metastatic melanoma are being offered a glimpse of hope in the form of immunotherapies, by targeting factors that foster the development and maintenance of an immunosuppressive microenvironment within tumors. Indeed, phase III clinical trials have revealed that therapies such as ipilimumab and pembrolizumab which target the CTLA-4 and PD-1 immune checkpoints, respectively, have significantly raised the survivorship of cancer patients (O'Donnell *et al.*, 2017).

Adoptive T-cell transfer (ACT) is a flexible and potent cancer strategy that can induce durable regression of several cancer types. One successful example is that metastatic melanoma patients who received tumor-infiltrating lymphocytes (TIL) have experienced complete and lasting tumor regression—and may be cured (Rosenberg *et al.*, 2011). Ongoing strategies to improve TIL therapy have been suggested by mouse models, human tissues and clinical trials. One focus of these studies is depleting negative regulatory cells, including myeloid-derived suppressive cells (MDSCs) and $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T (Treg) cells (Gros *et al.*, 2012; Yao *et al.*, 2012). The potency of TIL therapy can be enhanced by adding the flexibility to specifically target tumor antigens via antigen receptor gene engineering with CARs. Chimeric antigen receptors (CARs, also known as chimeric T-cell receptors or CAR-T) are engineered receptors, which are constructed of single-chain variable fragments (scFv) joined with TCR and T-cell co-stimulatory receptor domains. Engineered CAR-T cells are capable of recognizing intracellular tumor antigen, which is crucial to the increased application of cellular therapies. As an extension of TIL therapy, CAR T-cell therapy has been tested in

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various clinical trials against a wide range of tumor antigens. So far the most promising results from gene therapy with CAR T cells is with anti-CD19 CAR targeting of B cell malignancies (Kochenderfer *et al.*, 2010, 2012). Although no other cell surface antigen has been reported with more specificity than normal B-cell lineage, certain cancer testis antigens (MAGEA1, MAGEC1, MAGEB2, SSX1, SSX2 and CTAG1B) are shown to be potential targets for CAR T-cell therapy (Hofmann *et al.*, 2008).

Cytokines are a broad group of proteins, which are released in response to diverse range of cellular stresses, including carcinogen-induced injury and inflammation. As the mixture of cytokines that is present in the tumor microenvironment shapes host immunity, therapeutic manipulation of the cytokine environment constitutes cytokine therapies to stimulate protective responses. The infusion of high doses of IL-2 mediates tumor regression in a minority of patients with renal-cell carcinoma (RCC) or melanoma. Although the toxicities of this approach ultimately proved limiting, it is noteworthy that some patients with disseminated disease achieved durable clinical benefits (Fyfe *et al.*, 1995). Systemic IL-12 elicits striking antitumor effects in mouse models, but clinical testing was abruptly curtailed due to unexpected severe toxicities (Atkins *et al.*, 1997). In addition, systemic GM-CSF confers some clinical advantages in melanoma, prostate cancer and pulmonary metastases, perhaps through immune stimulation (Anderson *et al.*, 1999).

Characteristics of Natural Killer Cells

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Natural killer (NK) cells are large granular bone marrow-derived lymphocytes serving as an important component of the innate immunity. They act as a 'rapid force' that attack transformed and/or tumor cells based on their ability to lyse tumor cells without prior immune sensitization (Vivier *et al.*, 2011). NK cells respond faster than T and B cells as they do not have to rearrange the T-cell receptor or the immunoglobulin genes to create a highly diverse repertoire of specificities. Instead, they recognize the target cells by employing 'missing-self recognition'. Furthermore, some genetic experiments in mice recently reveal that absence of NK cells results in spontaneous tumor development and induced primary oncogenesis (Hayakawa Y et al. 2006).

NK cell function is controlled by a balance of NK cell inhibitory and activating signals. Several inhibitory receptor families have been identified in mice and humans (Yokoyama *et al.*, 1995; Wilson *et al.*, 2000) including killer inhibitory receptors (KIRs) and NKG2A (Ljunggren and Malmberg, 2007). NK cell inhibitory receptors, including killer Ig-like receptor (KIR), immunoglobulin-like receptor (LIR) and Ly49, bind to self-MHC (major histocompatibility complex) class I molecules, and this binding results in profound inhibition of the NK cells. A wide variety of NK cell-activating receptors have also been found. In humans, major NK activating receptors include NKp30, NKp44 and NKp46 (collectively called natural cytotoxic receptors NCRs), NKp80, NKG2D, CD2 and DNAM-1. Spontaneous cytotoxic activity is mainly triggered by NKG2D, leukocyte adhesion molecule DNAX accessory molecule1 (DNAM-1), and natural cytotoxicity receptors. Specifically, NK cells use their activating receptors like NKG2D to recognize stress induced ligands on abnormal cells such as tumor cells. Human NKG2D ligands

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include MHC class I chain-related protein (MICA), MICB, ULBP and RAET1(Bauer, 1999). Mouse NKG2D ligands include retinoic acid early transcript 1(Rae1), histocompatibility 60 (h60) and mouse UL16-binding protein-like transcript (MULT1). Many tumor cell lines and primary tumors from diverse tissue origins express NKG2D ligands. For instance, many primary tumor isolates from carcinoma (lung, breast, kidney, prostate, ovary and colon), melanoma and some primary leukemia cells express MICA. About 75% of primary cutaneous melanoma isolates and 50% of metastatic melanoma lesions express MICA protein (Vetter *et al.*, 2002). It was hypothesized that oncogenes and tumor suppressor genes involved in the process of transformation regulate NKG2D ligand expression. In addition, because these ligands are not widely expressed on healthy adult tissue, NKG2D ligands may present a useful target for immunotherapeutic approaches in cancer. Novel therapies targeting NKG2D ligands for the treatment of cancer have shown pre-clinical success and are poised to enter into clinical trials (Diefenbach *et al.*, 2001).

A balance in signaling mediated by activating and inhibitory receptors tightly regulates NK cell function. Thus, loss or down-regulation of MHC class I can render cells susceptible to NK cell-mediated lysis when an activating ligand is engaged (Salih, Rammensee and Steinle, 2002; Holdenrieder *et al.*, 2006). More in general, in addition to loss or down-regulation of MHC class I, NK cell can be activated by various other stimuli, such as contact with dendritic cells(DC), binding of IgG immunocomplexes, direct engagement of NKR by stress-induced tumor-associated molecules or pathogen-derived products, and several cytokines such as IL-1, IL-2, IL-12, IL-15, IL-18, IL-21 and type I

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interferons (IFNs). Upon cytokine stimulation, NK cells become lymphokine-activated killer (LAK) cells that proliferate, produce cytokines such as IFN-γ, and up-regulate effector molecules such as adhesion molecules, NKp44, perforin, granzymes, Fas ligand (FasL), and TRAIL (Trinchieri, 1989; Bottino *et al.*, 2004; Mirandola *et al.*, 2004; Moretta *et al.*, 2004). Essentially the NK cell lineage has been considered for cancer eradication owing to its ability to kill a wide variety of tumor cells spontaneously while sparing normal cells, which takes place as: secretion of cytotoxic IFN-γ, perforin/granzyme-dependent necrosis of target cells and induced apoptosis of target cells through Fas/FasL or TRAIL signaling pathway (Smyth *et al.*, 1999).

Early 1980s clinical trials started introducing IL-2 activated NK cells in the treatment of heavily tumor-burdened patients with solid primary or metastasized cancers. Subcutaneous injection of NK-stimulating doses of IL-2 showed 15-30% positive effect in patients with advanced RCC or melanoma (Rosenberg *et al.*, 1993; Clark *et al.*, 2003; McDermott *et al.*, 2005). However, both RCC and melanoma patients showed a variable susceptibility to apoptosis induced by TNF ligand members. Also and unfortunately, IL-2 treatment is associated with life-threatening toxicity, essentially represented by capillary leak syndrome (Fehniger, Cooper and Caligiuri, 2002). Another limitation of this approach is the fact that IL-2 but not IL-15 activated NK cells increase their sensitivity to apoptosis when in contact with vascular endothelium, likely causing a decrease in NK cell migration toward the cancer area. IL-15 appears as more efficient than IL-2 in expanding the NK cell compartment because it promotes the survival of NK cells and protects NK cells from AICD (Waldhauer and Steinle, 2008). Thus more recently,

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strategies favoring IL-15 trans-presentation to NK cells have been proposed; alternatively, early-acting cytokines such as Flt3-L, SCF, and IL-7 can be used to enhance NK cell numbers.

Differently from IL-2 and IL-15, IL-12 can increase NK-cell mediated IFN- γ production by binding to IL-12 receptor (IL-12R), and IL-1 and IL-18 potentiate the effect of IL-12 by up-regulating the IL-12Rs expression on NK cell surface. A substantial amount of evidence has demonstrated a critical role for endogenously produced IFN-γ in promoting host responses to primary and transplanted tumors. IFN-γ exerts its effects on cells by interacting with a distinct high affinity receptor that is expressed on virtually all normal cell surfaces. IFN-γ has been shown to suppress tumor angiogenesis, and to induce TRAIL-/FasL-mediated cellular susceptibility to apoptosis in a variety of tumor cells (Cretney *et al.*, 2002). In addition, IFN-γ is also a major macrophage-activating factor capable of inducing in macrophages the capacity to non-specifically kill a variety of tumor targets (Schreiber RD et al. 1986; MacMicking J, et al 1997). More recent experiments further demonstrate that endogenously produced IFN-γ participates in preventing primary tumor development by acting on both the innate and adaptive immune responses against tumors (Street SE et al. 2001). In one experiment, MCA-induced sarcomas from IFN-γ insensitive mice that lack IFNGR1 chain were engineered for enhanced expression of two IFN-γ-inducible components of MHC class I processing and presentation pathway to evaluate whether IFN-γ functioned. The result of this study showed that enforced expression of syngeneic TAP1 in IFN- γ-insensitive sarcoma cells converted the aggressively growing tumors into ones that were rejected in naïve

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syngeneic mice, which defined one important IFN-γ-induced response in tumors that promotes tumor rejection. Moreover, the IFN-γ-dependent enhancement of tumor immunogenicity not only serve as a mechanism for increased host protection against tumor development, but may also induce type I immunity and counteract tumor escape mechanisms that are actively promoted by advanced cancer cells and regulatory T lymphocytes through the secretion of type II cytokines such as IL-10 and TGF-β. To this regard, IL-21 proves promising in the context of anti-tumor immunotherapy (Davis MR et al. 2015). IL-21, which can lead to an augmentation of NK effects, has been found to promote both the expression of genes associated with type I response and the terminal differentiation of the highly cytotoxic CD16+ NK cell subset, thus potentially directing ADCC against tumor cells.

More advanced tumor cells are able to evade immunosurveillance by elements of the innate immune system such as NK cells, by down-regulating or "shedding" certain NKG2D ligands like MULT1. Moreover, they also can avoid certain apoptosis pathway such as Fas/FasL by down-regulating its expression. Recent studies reported the design of anti-tumor activity of novel fusion protein approaches. One fusion protein is MULT1E/Fas_{TI}, which consists of extracellular domain of MULT1 and the transmembrane and intracellular domain of Fas. The fusion construct was transfected into the mouse pulmonary carcinoma cell line TC-1. In vitro cell culture studies demonstrated that the binding of NKG2D/Fc to MULT1E/Fas_{TI} expressed on tumor cells was able to elicit apoptosis and to activate NKG2D-expressing cells including NK cells. In vivo subcutaneous tumor studies demonstrated that tumor cells-expressing MULT1E/Fas_{TI}

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grew significantly slower than cells without the fusion protein. Pulmonary metastasis studies showed that most of the mice completely rejected tumor cells expressing MULT1E/Fas$_{TI}$ (Kotturi *et al.*, 2008, 2010). Another example of effective fusion protein is MULT1/mIL-12, which conjugated MULT1 (NKG2D ligand) with murine IL-12. Resulting fusion protein was transfected into TC-1 cell line. RT-PCR and fluorescent microscopy result showed a significant level of MULT1/mIL-12 secretion, NK cell activation and IFN-γ production and cytotoxicity within tumor cell micro-environment (Tietje *et al.*, 2017). The results demonstrate that the fusion protein treatment with engagement of NK cells and other immune cells can activate NK cells through NKG2D receptor or IL-12 receptor to directly lyse the tumor cells and exert bi-functional against tumor cells.

Immunology of Interleukin-12

Interleukin-12 (IL-12), known as NK cell stimulatory factor 2, is an activator and initiator of NK cell proliferation (Robertson, et al. 1992; Rodolfo M, et al. 1999). It was identified as a heterodimeric cytokine composed of two covalently linked p35 and p40 (Kobayashi M et al. 1989). IL-12 binds to the IL-12 receptor, which is a heterodimeric receptor formed by IL-12R-β1 and IL-12R-β2. Upon binding, IL-12R-β2 becomes tyrosine phosphorylated and provides binding sites for kinases, Tyk2 and Jak2 (Wang KS et al. 2000). These are important in activating critical transcription factor proteins such as STAT4 that are implicated in IL-12 signaling in T cells and NK cells. Initial characterization of its biological activities revealed that IL-12, when added to human

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peripheral blood lymphocytes, induced IFN-γ production, increased NK cell cytotoxicity as well as T cell proliferation in response to mitogenic lectins and phorbol-diesters. Subsequent studies indicated that IL-12 could boost the generation of cytotoxic T cells by promoting the transcription of genes encoding cytolytic factors including perforin and granzymes.

A number of biological properties of human IL-12 have been evaluated in vitro. Among its properties are the ability to act as a NK cell and T cell growth factor, to enhance NK/LAK cell cytolytic activity, to augment cytolytic T cell responses and to induce secretion of cytokines, particularly IFN-γ, from NK and T cells (Kobayashi M et al. 1989; Gately et al. 1991; Robertson MJ et al.1992). Since both T and NK cells have been implicated as antitumor effector cells and IFN-γ has been shown to have antitumor activity, IL-12 was believed potential to be used as an immunomodulatory cytokine in the therapy of malignancies. More recent studies demonstrated that the heterodimeric cytokine IL-12 is a key mediator of both innate and cellular immunity with potent anti-tumor and anti-metastatic activity by majorly interacting with its receptor. (Tsung K et al. 1997; Rodolfo M et al. 1999). IL-12 also contributes to activation of CD8+ T cells in absence of T cells and NK cells. (These antitumor and anti-metastatic activities of IL-12 have been extensively shown in murine models including melanomas, mammary carcinoma, colon carcinoma, renal carcinoma and sarcomas (Colombo and Trinchieri, 2002). Recombinant IL-12 given systemically had powerful antitumor activities in murine models that led to its clinical development, which began in 1994. In preclinical models of cancer, the activity of IL-12 has been investigated in patients with advanced

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solid tumors and hematologic malignancies, as either monotherapy or in combination with other treatment. In Phase I clinical trials designed in an intrapatient dose escalation fashion, it was not realized that initial low doses of IL-12 resulted in desensitization against the biological effects of IFN-γ produced upon subsequent IL-12 doses. Intravenous (i.v.) doses, which were tolerated in Phase I, caused severe toxicity in Phase II clinical trials as many other cytokines and thus put a stop to the clinical development of this active antitumor agent for several years. Targeted delivery of IL-12 to the tumor environment or gene transduction of malignant cells with IL-12 genes is expected to increase the therapeutic index of the cytokine. Efforts have been given to generate local high dose of IL-12 therapy. In one study, local high doses of IL-12 were achieved by a glycosylphosphatidylinositol (GPI)-anchoring technology. GPI-anchored IL-12 was successfully expressed on the cell surface as indicated by FACS analysis and IL-12 ELISA assay, which enhanced lymphocyte infiltration and significantly inhibited tumor growth. More importantly, when GPI-anchored IL-12 and GPI-anchored IL-2 were co-delivered, a synergistic anti-tumor effect was observed in both subcutaneous and intravenous tumor models (Jianfei Ji et al. 2004). Gene encoding lymphocyte attracting chemokines can be successfully combined with IL-12 therapy. This approach has shown remarkable efficacy with adenoviruses encoding the chemokines lymphotactin and IP-10 (Emtage PC et al. 1999). Therefore, a general mechanism of action for synergistic combinations has been proposed in which a halt in tumor growth by angiogenesis inhibition and NK activation trailblazer the route for artificially boosted T cell responses.

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Gene transfer of IL-12 into tumors is now entering clinical testing and it is hoped that the safety records will be as good as the preclinical data.

Fas Mediated Apoptosis Pathway

Fas receptor (Fas), also known as apoptosis antigen 1 (APO-1), cluster of differentiation 95 (CD95), is a transmembrane cell-surface death receptor that belongs to the tumor necrosis factor (TNF) receptor superfamily. Fas contains three cysteine-rich extracellular domains at the N-terminus, which are responsible for ligand binding, the transmembrane domain, and an intracellular death domain (DD) of about 80 amino acids that is essential for transducing the apoptotic signals (Peter et al. 2003). Binding of Fas ligand (FasL or CD95L) to Fas receptor results in aggregation of the receptor molecules and recruitment of the adapter molecule, Fas-associated death domain (FADD), through DD-DD interactions. Fas forms the death-inducing signaling complex (DISC) upon ligand binding. Membrane-anchored Fas ligand trimer on the surface of an adjacent cell causes oligomerization of Fas. Recent studies which suggested the trimerization of Fas could not be validated. Other models suggested the oligomerization up to 5-7 Fas molecules in the DISC. FADD also contains a death-effector domain (DED), which facilitates binding to the DED of FADD-like interleukin-1 beta-converting enzyme (FLICE), more commonly referred to as caspase-8. Active caspase-8 is then released from DISC into the cytosol, where it cleaves effector caspases such as caspase-3 that in turn cleave a restricted set of target proteins and are responsible for apoptosis in the cell (Houston et al. 2004).

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Fas ligand is predominantly expressed in activated T lymphocytes and natural killer cells (Nagata et al 1997). Likely, the most established pro-apoptotic activity of Fas is to mediate the apoptotic death of either virus-infected or cancer cells when engaged by a CD8+ cytotoxic lymphocyte (CTL). In addition to the perforin/granzyme pathway and some indirect mechanisms involving cytokines such as tumor necrosis factor α (TNF α) and IFN-γ, Fas/FasL is a direct major system that both CTL as well as CD4+ cytolytic effector T cells use to eliminate neoplastically transformed cells (Lowin et al. 1994; Rouvier et al. 1993; Stalder et al. 1994). Tumor cells have developed many strategies for escaping immune surveillance. One of these strategies is that tumor cells can escape from Fas-mediated apoptosis by decreasing surface expression of Fas *[Moller P et al. 1994; Ivanov VN et al. 2003]*. The CD95 apoptotic signal can also be inhibited at the level of the DISC via increased expression of cFLIP (cellular FLICE inhibitory protein), which can inhibit the interactions of caspase-8 and -10 with the DISC (Irmler et al. 1997), or via reduced expression of FADD (Tourneur et al. 2003) or caspase-8 (Teitz et al. 2000). Loss of apoptosis signaling through CD95 can also be the consequence of deregulation of the expression of the Bcl-2 family members (Igney et al. 2002), and downregulation and mutating pro-apoptotic genes such as BAX, APAF1 or Fas (Teitz et al. 2000), thereby favoring tumor survival.

Apoptosis induction is the most well-established activity of CD95, documented and summarized in numerous articles. In the context of cancer, it is relevant that CD95L is one of only a few molecules that immune cells use to activate apoptosis pathways to eliminate cancer cells (Strasser et al. 2009). Apoptosis induction as a cancer cell killing

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strategy is presumed to be accomplished by tumor-infiltrating lymphocytes expressing CD95L. Apoptosis induction in cancer cells through CD95 is the only scenario in which recombinant CD95L could be used for cancer therapy. However, given the fact that almost all established cancers express CD95 and most cancer cells are resistant to apoptosis induction, stimulating CD95 on cancer cells may not be an effective approach to killing cancer cells. In addition, stimulation of CD95 could never be used therapeutically because of major side effects such as massive apoptosis induction in the liver (Ogasawara et al. 1993). In one present study, researchers intended to combine the NK cell-mediated cytolysis and Fas-mediated apoptosis into one fusion protein by using the extracellular domain of MULT1 and the transmembrane and intracellular domains of Fas in a mouse model. The engagement of NK cells and/or other immune cells with tumor cells expressing the fusion protein succeeded in not only sending an apoptotic signal to the tumor cells via Fas-induced mechanisms but also lysing directly by the activated NK cells. In this case, it is hypothesized that binding of MULT1 to NKG2D could contribute to the trimerization of Fas, although it has not been confirmed. The results demonstrated that this fusion protein exerted bi-functional anti-tumor effects *in vitro* and *in vivo* (Kotturi *et al.*, 2008, 2010).

In 2004, it was reported that stimulation of CD95 on 22 apoptosis-resistant cancer cell lines increase their motility and invasiveness in vitro [Barnhart BC et al. 2004]. It was demonstrated in various cancer cell lines that CD-95 mediated invasiveness requires activation of NF-κB and ERK, and involves active caspase-8 and urokinase plasminogen activator (Barnhart et al. 2004). It is now widely accepted that once cancer cells acquire

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resistance to CD95-mediated apoptosis, further stimulation of CD95 is tumorigenic (Lee et al. 2003; Kleber et al. 2008; Nijkamp et al. 2010). Following up on the findings that CD95 contributes to the proliferation of cancer cells, it was also reported that the elimination of either CD95 or CD95L kills cancers (*in vitro* and *in vivo*) in a process termed as DICE (death induced by CD95 or CD95L elimination) (Hadji et al. 2014). The activity of CD95 as a survival factor seems to be mostly relevant to cancer cells, as none of the normal tissues during embryonic development in either CD95 or CD95L knockout mice showed a growth defect. Consistently, it was demonstrated that blockade of Fas signaling in breast cancer cells suppressed tumor growth and metastasis (Liu et al. 2013). In addition, in two ovarian cancer mouse models and one mouse model of chemically induced liver cancer, tumor formation was severely reduced in the absence of CD95 (Chen et al. 2010; Hadji et al. 2014).

In conclusion, it is clear that up-regulation of wild-type Fas on the surface cells can result in programmed cell death by the Fas/FasL pathway. Given the fact that tumor cells may have exploited the presence of FasL on their cell surface to evade deletion by Fas-bearing tumor-specific cytotoxic T cells, enhanced Fas expression in cells which can be induced by cytotoxic drugs or via gene therapy is currently in therapeutic use.

Fusion Protein Based Bi-functional anti-tumor effects

Fusion proteins or chimeric proteins (literally, made of parts from different sources) are proteins created through the joining of two or more genes that originally coded for separate proteins. Translation of this fusion gene results in a single or multiple

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polypeptides with functional properties derived from each of the original proteins. Recombinant fusion proteins are created artificially by recombinant DNA technology for use in biological research. A recombinant fusion protein is created through genetic engineering of a fusion gene. This typically involves removing the stop codon from a cDNA sequence coding for the first protein, then appending the cDNA sequence of the second protein in frame through ligation or overlap extension PCR. A linker sequence between two entities is often added, which makes it more likely that both proteins fold more independently and behave different biological functions as expected. The purpose of creating fusion proteins in drug development is to impart properties from each of the "parent" proteins to the resulting chimeric protein. Several chimeric protein drugs are currently available for medical use.

Most cytokines act as soluble factors that bind and activate receptors on target cells (Schooltink et al.2002; Vilcek et al. 2004). Consequently, therapeutic use of cytokines for cancer therapy, for example by intravenous applications, requires that the cytokines reach the tumor and accumulate there in order to execute their immune-modulating or cytotoxic activities. Importantly, delivery of the cytokines into the vicinity of the tumor cells in most cases should be sufficient for the induction of a stimulatory activity on immune cells. Therefore, various cytokines have been approved for therapy of cancer and other diseases and many more are under development in the past years (Ortiz-Sánchez et al. 2008).

IL-2 fusion proteins

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IL-2 is a pleiotropic and potent stimulator of the immune system including the activation of natural killer (NK) cells, monocytes and cytotoxic T cells. IL-2 can act directly by inducing apoptosis of tumor cells, but also indirectly by activation of cytotoxic cells, e.g. CTLs, NK cells, and macrophages, including the upregulation of MHC class II molecules (Malek and Castro 2010). IL-2 has been studied extensively since almost two decades for the generation of antibody-cytokine fusion proteins against cancer progression. From clinical trials, toxicity, especially vascular leakage syndrome, associated with therapeutic doses as well as a short serum half-life was identified as the two major problems that limit the therapeutic use of IL-2 (Harvill and Morrison 1995). These issues demonstrated that a target-dependent accumulation of IL-2 and local immune response and improved pharmacokinetics is required. For example, IL-2 was used to generate an F(ab')-IL-2 fusion protein directed against carcinoma cells (Fell et al. 1991). In a variety of fusion proteins, the cytokine was fused to C-terminus of the antibody heavy chain. Various IgG-IL-2 fusion proteins based on either IgG1 or IgG3 established that these proteins exhibit a combination of biological properties including binding to tumor cells, Fc-mediated effector functions and stimulation of IL-2R expressing lymphocytes (Harvill and Morrison 1995, 1996; Gillies et al. 1992). In further studies, cooperative activity of IL-2 with other cytokines was demonstrated by a combination of anti-CD30, single-chain-Fv-Fc-IL-2 (scFv-Fc-IL-2) and scFv-Fc-IL-12 fusion proteins, which resulted in enhanced activation of resting NK cells and tumor cell lysis (Heuser et al. 2004; Hombach et al. 2005).

IL-12 fusion proteins

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IL-12 is a pro-inflammatory cytokine which induces proliferation of NK and T cells, production of other cytokines, e.g. IFN-γ and activation of effector T cells. Therefore, IL-12 has a central role in the induction of type I cell-mediated immune response in cancer and inflammation (Xu et al. 2010). Based on studies with IL-2 fused to an antibody heavy chain, a similar fusion protein was generated utilizing IL-12 (Gillies et al. 1992). Because IL-12 is a heterodimer, the production of the IgG-IL-12 fusion protein involved co-expression of the antibody light chain together with the p40 unit, and the p35 unit fused to the C-terminus of the heavy chain. ScIL-12 was used to generate a variety of different antibody-IL12 fusion proteins, e.g. by fusing mouse scIL-12 to the N-terminus of an anti-HER2 IgG3 heavy chain antibody, and antitumor activity was observed in syngeneic models of HER2-transfected CT26 tumor cells (Peng et al. 1999). When combining the anti-CD30 scFv-Fc-scIL-12 fusion protein with an anti-CD30 scFv-Fc-IL-2 fusion protein, a cooperative activation of resting NK cells, induction of IFN-γ secretion and enhanced target cell lysis was observed *in vitro* (Hombach et al. 2005).

In other cases, cytotoxic fusion proteins for tumor therapy are composed of an antibody -based targeting moiety and an effector molecule. Effectors may possess enzymatic activity conferring cytotoxicity after internalization or be an antibody-targeted death-receptor ligand that induces apoptosis after interaction with a death receptor (DR). Fusion proteins makes use of cell-death inducing ligands such as tumor-necrosis factor, tumor necrosis factor α-related, apoptosis-inducing ligand Fas ligand and a tumor-targeting antibody moiety. Cytotoxic fusion proteins based on caspase 6 have been explored. Expression of activated caspases in tumor cells would bypass situations in

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which the caspase activation process is inhibited by anti-apoptotic proteins such as inhibitors of apoptosis (IAPs) (Straub et al. 2011). Death receptors (DRs) are members of the TNF-receptor (TNFR) superfamily with functions in regulation of survival and cell death, differentiation and immunity. Up to now a total of 18 ligands and 28 receptors have been identified including TRAIL and Fas (CD95), and almost all of the receptors and ligands are transmembrane proteins (Ashkenazi 2002). DRs are expressed on many types of tumors and this finding can be exploited to induce regression of tumors by treatment with their cognate ligands. After receptor ligand interaction, a death-inducing signaling complex (DISC) is assembled through the adapter protein FAS-associated death domain (FADD), leading to recruitment and activation of caspase 8 and/or caspase 10. DRs can be engaged with recombinant trimerized ligand (TRAIL, TNF, and FASL) or with agonistic antibodies binding to the extracellular domain of the receptors with and without the requirement for cross-linking of the receptors.

Based on the overview above, It can be concluded that (i) cancer is a leading cause of death worldwide with capability to accumulate mutation and escape immunological killing; (ii) NK cells play a significant role in controlling tumorigenesis mainly through cytotoxic killing of tumor cells; (iii) delivery of IL-12 will activate NK cells, reshape the tumor environment and induce tumor elimination; (iv) Fas is one of most important death receptors which can induce Fas/FasL-mediated apoptosis pathway and kill targeted cells; (v) the strategy of chimeric fusion proteins demonstrate more effective and promising in various diseases including cancer via a combination of different functional components.

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Lentivirus Based Gene Delivery System

The concept of gene delivery vectors based on retroviruses was introduced in the early 1980s by Mann et al (Mann et al. 1983). Retroviral vectors, such as those based on murine leukemia virus (MLV), are attractive vehicles for delivering genes especially in view of inducing anti-tumor responses due to their relatively large coding capacity, efficient gene transfer and lack of virus-encoding proteins which could elicit undesirable immune responses (Breckpot et al. 2007). Moloney Murine Leukemia Virus (MLV) has a simple genome; from this genome, the polyproteins, *gag*, *pol* and *env* are required in *trans* for viral replication and packaging. Required in *cis* are the 5' and 3' long-terminal repeat (LTR), the integration sequences as well as the packaging site (ψ), the transport RNA-binding site and finally additional sequences involved in reverse transcription. To generate replication-deficient retroviral vectors, the *gag*, *pol* and *env* genes are replaced with an expression cassette.

The major disadvantages of MLV vectors, however, are their inability to transduce non-diving cells, many of which are clinically relevant target **(**Lewis et al. 1994; Miller et al. 1990**)**. These oncogenic retroviral vectors are also associated with limitations such as low viral titers and the instability of viral particle (Doux, et al. 1999). To overcome these shortcomings, lentivirus-based vectors were developed. Lentiviruses are a subgroup of the retrovirus family, which have been developed mostly from simian immunodeficiency virus (SIV) and human immunodeficiency virus type I (HIV-1). Unlike retroviruses, lentiviruses are capable of transducing quiescent cells. Their

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genomes are slightly more complicated, containing accessory genes that regulate viral gene expression, control the assembly of infectious particles, modulate viral replication in infected cells and contribute to the persistence of infection (Kay et al. 2001).

The design of lentivirus-based vectors is based on the separation of *cis*- and *trans*-acting sequences. Generally, lentiviral particles are generated through transient transfection of three plasmids in human embryonal kidney 293 FT (Naldini et al. 1996). The plasmids in question are: (1) a packaging plasmid, (2) a transfer plasmid, (3) an envelope-encoding plasmid. Nuclear import of the transfer construct was improved by including the central polypurine tract (cPPT) and its central termination sequence (CTS), together forming a triple helix (TRIP). This structure mediates the transport of the pre-integration complex through the nuclear pores. cPPT/CTS-containing transfer vectors yields higher virus titers and proved enhanced transgene expression in the target cell (Follenzi, et al. 2000). Addition of post-transcriptionally active elements, such as the woodchuck hepatitis B post-transcriptional regulatory element (WPRE), represents another strategy to improve the lentiviral vector design. WPRE may improve gene expression by modification of polyadenylation, RNA export or translation (Zuffery et al. 1999). The third generation of lentivirus-based vector, containing both cPPT and WPRE, has demonstrated to yield more than 4 fold of viral titers.

Among the first and still most widely used glycoproteins for pseudotyping lentiviral vectors is VSV.G. Lentiviral vectors pseudotyped with VSV.G offer significant advantages in that VSV.G appears to interact with a ubiquitous cellular receptor on cells,

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endowing the vector with a broad host-cell range. Furthermore, VSV.G confers high vector particle stability, allowing downstream processing of the viral particles.

Although lentiviral particles have been widely used in pre-clinical and clinical trials, large-scale and high-titer vector production is challenging, due to the lack of simple procedures for rapidly processing large volumes of cell culture supernatant. Traditionally, ultracentrifugation of virus-containing cell culture supernatants has been used for this purpose (Sena-Esteves et al. 2004; Coleman, et al. 2003). However, these approaches are limited in terms of their capacity to handle large volumes, thus making this procedure tedious. Thus, there is an emerging need for quick, reproducible and less laborious procedures that rapidly reduce the volume of the cell culture supernatant to be processed. Pham et al and Zhang et al have both described a precipitation method to concentrate lentiviral vectors involving the co-precipitation of viral supernatants with calcium phosphate and poly-L-lysine, respectively (Pham et al. 2001; Zhang et al. 2001). More recently, a lot more lentivirus precipitation solutions have been developed to optimize the purification and concentration of lentiviral particles. These lentivirus precipitation solutions majorly consist of a mixture of polymer, such as Poly (ethylene glycol). Additionally, methods based on anion exchange chromatography of HIV-1 vectors pseudotyped with VSV.G and baculovirus glycoprotein 64 have been successfully established (Schauber et al. 2004).

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CHAPTER TWO

AIMS OF THIS STUDY

Whereas cytokine therapy for cancer treatment demonstrated effective in activating immune response against tumor cells, one major obstacle with the use of these cytokines is their severe side effects when delivered systemically at high doses. Another obstacle is tumor cells evade immunosurveillance of the innate immune system such as natural killer (NK) cells as well as of the Fas-mediated apoptosis by down-regulating its expression. To reshape the immune response in the tumor microenvironment, therapies that introduce factors to alter the endogenous tumor immunity have been developed using mAb, cytokines and fusion proteins. In the previous study I report the design and preliminary evaluation of the antitumor activity of a novel fusion protein—mIL-12/Fas_{TI}, consisting of the extracellular domain of mIL-12 and the transmembrane and intracellular domain of Fas. The fusion construct (mIL-12/Fas_{TI}) were transfected into the mouse pulmonary carcinoma cell line TC-1, and stable cell clones expressing the fusion protein were selected as assayed by RT-PCR and immunohistochemistry (IHC). It was well demonstrated from in vitro biological function assays that fusion protein IL12/Fas_{TI} successfully activated NK92 cells and peripheral blood mononuclear cells (PBMCs) with enhanced production of IFN-γ and overall cytotoxicity against target tumor cells. Induction of apoptosis within target tumor cells expressing IL12/Fas_{TI} was also confirmed through caspase 3 activity detection and Annexin-V staining. The result of preliminary *in vitro* study appears to be promising; therefore, to further apply the strategy

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to a human setting, an efficient gene delivery system is in demand for cancer gene therapy.

1. The construction of an expression vector containing the mIL12/FasTI fusion gene

To avoid systemic toxicity of traditional IL-12 treatment and to realize the high concentration of IL-12 in the tumor environment, our mIL-12 control plasmid will include the cDNAs of *mIL-12* as well as an anchoring sequence so that mIL-12 protein can be anchored on the cell surface once expressed successfully. Furthermore, Fas transmembrane and intracellular domain (FasTI) will be conjugated to mIL-12 to create the fusion gene $mIL\text{-}12/Fas_{TI}$, which is hypothesized to exert bi-functional anti-tumor elimination by both NK cell cytotoxicity and Fas-mediated apoptosis. The cDNAs of *mIL-12* and the transmembrane/intracellular domains of Fas will be cloned into expression vector pcDNA3.1/Zeo (+). Meanwhile, a control vector containing cDNA of mIL-12 and cell membrane anchoring sequence will be produced.

2. Transfection and selection of stable clones expressing the mIL12/FasTI fusion protein

To quickly evaluate if the fusion gene construct is correct at both protein and RNA levels, the resulting plasmid will first be used to transfect TC-1 mouse lung carcinoma cells using Lipofectamine2000. Stable clones will be selected and the expression of the fusion gene product will be confirmed at RNA level by RT-PCR and protein level by immunohistochemistry.

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3. *In vitro* biological functions of the mIL12/FasTI fusion protein

To determine if the fusion protein clones are able to exert anti-tumor functions effectively, two major parts of in vitro assays will be performed: the activation of NK cells and tumor cell apoptosis. To detect if NK cells will be activated, the production of IFN-γ will be detected by using human IFN-γ ELISA assay kit. The cytotoxicity of NK cells will be performed by MTS cell proliferation assay.

4. Construction of Lentivirus-based gene expression vectors

To achieve the high production of lentiviral particles, the cDNAs of mIL-12 and the transmembrane/intracellular domains of Fas will be cloned into lentivirus based expression vector. Meanwhile, control vectors encoding the cDNAs of mouse IL12, Fas, and LacZ will be established following the similar methodology.

5. Production of lentivirus in 293 FT cells

To produce the lentiviral particles containing the fusion gene of interest, lenti-constructs (pLenti-IF/IL12/Fas/LacZ) as well as 3 packaging plasmids (pLP1, pLP2 and pLP/VSVG) will be used to transfect 293 FT cell line using lipofectamine 2000. The viral titer of concentrated viral particles will then be evaluated using Lenti-X p25 rapid titer assay and GFP-based cytometry.

6. *In vitro* expression and biological functions of the virally transduced fusion clones

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Once the viral titer is estimated, viral particles will be used to transduce tumor cells. The transient protein expression level of each lenti-construct will be demonstrated by RT-PCR and IHC. Different virally transduced clones (Lent-IF, Lent-IL12, Lent-Fas) will be co-cultured with human PBMC and assayed for human IFN-γ ELISA, caspase 3 activity, Annexin-V apoptosis and overall cytotoxicity against tumor proliferation.

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CHAPTER THREE

MOUSE INTERLEUKIN-12/FAS$_{TI}$: A NOVEL BI-FUNCTIONAL FUSION PROTEIN FOR CANCER IMMUNO/GENE THERAPY

Abstract

Whereas cancer immunotherapy with cytokines in recent researches demonstrated effective in activating immune response against tumor cells, one major obstacle with the use of these cytokines is their severe side effects when delivered systemically at high doses. Another challenge is that advanced tumor cells often evade immunosurveillance of the immune system as well as of the Fas-mediated apoptosis by various mechanisms. In the present study, we report the design and preliminary evaluation of the antitumor activity of a novel fusion protein—mIL-12/Fas$_{TI}$, consisting of mouse interleukin-12 and the transmembrane and intracellular domains of mouse Fas. The fusion construct (pmIL-12/Fas$_{TI}$) was transfected into mouse lung carcinoma cell line TC-1. Stable cell clones expressing the fusion protein were established as assayed by RT-PCR and immunohistochemistry. ELISA and cell proliferation analyses demonstrated that NK cells were effectively activated by the fusion protein with increased IFN-γ production and cytotoxicity. Enhanced caspase 3 activity of the clones when co-cultured with NK cells indicated that apoptosis was induced through Fas/FasL signaling pathway. The preliminary results suggest a synergized anti-cancer activity of the fusion protein. It may represent a promising therapeutic agent for cancer treatment.

Keywords: Interleukin-12; Fas; natural killer cell; apoptosis

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Introduction

Natural killer (NK) cells are an important component of the innate immune system that provides rapid responses to malignantly transformed or infected cells through immunosurveilance. NK cells also help to shape the adaptive immunity by interacting with dendritic cells and producing the key cytokine IFN-γ. The NK cell lineage has been considered for cancer eradication owing to its ability to kill a wide variety of tumor cells spontaneously while sparing normal cells (Smyth *et al.*, 1999, 2005). Advanced tumor cells, on the other hand, are able to inhibit NK cells and other anti-tumor immune cells in a tumor microenvironment, where the cytokine profile favors tumor growth. One of the challenges ahead of cancer immunologists is how to reshape the tumor microenvironment to favor killer cells, such as NK cells, cytotoxic T lymphocytes (CTLs), γδT cells, etc.

IL-12, also known as NK cell stimulatory factor 2, is an activator and initiator of NK cell activation (Robertson et al. 1992; Rodolfo et al. 1999). One of IL-12's major functions is to enhance NK cell-mediated IFN-γ production, which promotes host responses against tumors and regulates tumor development by interacting with its receptor. IL-12 also contributes to activation of CD8+ T cells in absence of helper T cells and NK cells. IL-12 has, therefore, been selected as a good candidate for cancer cytokine therapies. In experimental tumor models, recombinant IL-12 treatment has a dramatic anti-tumor effect on transplantable tumors, chemically induced tumors, and in tumors arising spontaneously in genetically modified mice. IFN-γ and a cascade of other pro-inflammatory cytokines induced by IL-12 have a direct toxic effect on the tumor cells or

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may activate potent anti-angiogenic mechanisms (Colombo and Trinchieri, 2002). However, IL-12's systemic high toxicity hindered its clinical applications (Atkins *et al.*, 1997; Car *et al.*, 1999). Efforts have been given to generate local high dose of IL-12 therapy. Previous studies have successfully extended the GPI (glycosylphosphatidylinositol) anchor technology to murine IL-12. A fusion gene consisting of murine IL-12 cDNA and a GPI-anchoring signal from decay accelerating factor (DAF) showed significant synergistic anti-tumor effect when co-expressed with GPI-anchored IL-2 (Ji *et al.*, 2002, 2004).

Fas (CD95) is a transmembrane cell surface death receptor that belongs to the tumor necrosis factor (TNF) receptor superfamily. Fas contains three cysteine-rich extracellular domains at the N-terminus, which are responsible for ligand binding, the transmembrane domain, and an intracellular death domain (DD) that is essential for transducing apoptotic signals (Peter et al. 2004). Binding of Fas ligand to Fas receptor results in the recruitment of Fas-associated death domain (FADD) through DD-DD interactions. The death-effector domain of FADD then recruits pro-caspase-8/10 to the receptor, resulting in the formation of death-inducing signaling complex (DISC). DISC activates effector caspases such as caspase-3 that in turn cleaves a restricted set of targets proteins and is responsible for apoptosis in the cell. (Houston et al. 2004)

In this study, we intended to combine the NK cell-mediated cytotoxicity and Fas-mediated apoptosis into one fusion protein by constructing a fusion gene containing cDNAs encoding mouse *IL-12* followed by the transmembrane and intracellular domains

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of *Fas*. We hypothesized that the tumor cells expressing the fusion protein will not only send an apoptotic signal to tumor cells but also activate the NK cells through IL-12 receptor. Therefore, not only the engaged tumor cells will be killed via Fas-induced mechanisms, the neighboring tumor cells that do not express the protein will also be killed by activated NK cells (Kotturi *et al.*, 2008, 2010).

Materials and Methods

Cells

The mouse lung carcinoma TC-1 cells (ATCC No. CRL-2785) were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 100μg/ml gentamicin at 37℃ with 5% CO2.

The human natural killer cell line NK92 cells (ATCC No. CRL-2407) were cultured in alpha MEM containing 12.5% fetal bovine serum, 12.5% horse serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.02 mM folic acid, 0.1 mM 2-MercaptoethanoL, 100 μg/ml gentamicin and 4 ng/ml interleukin-2 at 37℃ with 5% CO_2.

Construction of pcDNA3.1 (+) mIL-12/Fas_{TI}/Zeo Vector

Mouse IL-12 cDNA was cloned from pORF-mIL-12 (InvivoGen, CA, USA), and the transmembrane/intracellular domains of Fas were amplified from a previous pcDNA3.1 (+) MULT1E/Fas_{TI}/Zeo expression vector using 5' primer (CGGGATCCCCCAGAAATCGCCTATGGTTGT TGACC) and 3' primer (CGGAATTCTCACTCCAGACATTGTCCTTCATTTTC). To allow a continuous open

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reading frame the mIL-12 stop codon was removed from the 3' end of the sequence during PCR amplification and replaced with a (GGGS)$_3$ linker sequence (GGTGGTGGTTCTGG TGGTGGTTCTGGTGGTGGTTCT) to separate the two functional domains of fusion protein to maintain original folding and binding ability of the proteins. HindIII, BamHI, EcoRI restriction cutting sites were designed for 5'end of mIL-12, 3'/5' ends of mIL-12 and Fas$_{TI}$ and 3'end of Fas$_{TI}$, respectively. The primers are: 5'IL-12: CCAAGCTTCCATGGGTC AATCACGCTACCTCC; 3'IL-12: CCAGAACCACCACCGGATCGGACCTGCAGGGAACACATGC;5'FasTI: CGGGATCCCCCAGAAATCGCCTATGGTTGTTGACC;3'FasTI: CGGAATTCTCACTCCAGACATTGTCCTTCATTTC

The sequence of construct was confirmed using DNA Sanger sequencing.

Construction of pcDNA3.1(+)/ mIL-12/Zeo Vector (containing PDGRF transmembrane domain)

In order to create a cell membrane anchored form of moue IL-12, the IL-12 sequence was amplified from pORF-mIL-12 using 5'primer (CCCCCCGGGAGGGTCATTCCAGTCTCT) with XmaI restriction cutting site and a 3' primer (TCCCCGCGGGGATCGGACCCTGCAG GGAAC) with a SacII restriction cutting site by PCR. The fragments were excised and gel purified using a gel purification kit (Qiagen, Valencia, CA, USA). Double enzyme digestion was performed on the purified fragments of mIL-12 and pDisplay vector (Invitrogen life technologies) using XmaI and SacII allowing for the insertion of mIL-12 into the vector. The enzyme

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digested mIL-12 fragment and pDisplay vector fragment were ligated to create the plasmid pDisplay (+) mIL-12. In order to make the expression vector zeocin resistant, restriction enzymes HindIII and XhoI were used to cut plasmid pDisplay (+) mIL-12 and fragment containing mIL-12 sequence together with the cell membrane anchoring sequence was retrieved and inserted into plasmid pcDNA3.1/Zeo(+) to have plasmid pcDNA3.1(+)/mIL12/Zeo.

The sequence of construct was confirmed using DNA Sanger sequencing.

Transfection of cells

TC-1 cells were transfected with pcDNA3.1 (+) mIL-12/Fas_{TI}/Zeo or pcDNA3.1 (+) mIL-12/ Zeo using Lipofectamine 2000 (Invivogen) as directed by the manufacturer. To obtain stable clones expressing fusion protein or control protein, the transfected TC-1 cells were cultured in selecting medium containing 200µg/ml zeocin. Drug-resistance clones were collected and sub-cultured in the presence of the selective drug.

RT-PCR

Total RNA was extracted from each clone using an RNeasy Plus from Qiagen following the manufacturer's directions. cDNAs were produced from 2µg of the total RNA using the QuantiTect RT kit from QIAgen following the manufacturer's directions. Five microliters of the cDNA product were added to a PCR reaction containing primers amplifying a 1059 bp portion of mIL-12/Fas_{TI} fusion gene sequence containing 522bp of the mIL-12, the linker (GGTGGTGGTTCTGGTGGTGGTTCTGGTGGTGGTTCT, and

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the entire 495-bp of FasTI. A Phusion High-Fidelity DNA polymerase kit was used with a 5' primer GCAGTGACATGTGGAATGGC and a 3' primer CGGAATTCTCACTC CAGACATTGTCCTTCATTTTC. Similar reaction was also carried out to amplify portion of mIL-12 sequence using 5' primer GGAAGCACGGCAGCAGAATA and 3' primer AACTTGAGGGAGAAGT AGGAATGG to amplify a 180-bp fragment of mIL-12. Beta-actin housekeeping gene was also amplified as controls.

Immunohistochemistry (IHC)

Glass slides with cells of Clones IL-12/8, IL12-Fas/10 (IF-10) and TC-1 were prepared using cytospin technology. A hundred thousand cells in 1 ml PBS were transferred into assembled cytospin cassettes and centrifuged in the CytoTek centrifuge at 2,000 rpm for 10 minutes at room temperature. After centrifuge the cells on the slides were fixed with 4% para-formaldehyde (PFA) solution for 10 minutes. The cells were then stained at room temperature for one hour with isotype rat IgG2a antibody (R&D Systems, clone #54447) or anti-mouse IL-12/p35 antibody (R&D Systems, clone #45806) after a 1:500 dilution with sterile PBS. After washing, secondary antibody conjugated with HSS-HRP was added to the cells and incubated for 30 minutes at room temperature. DAB substrate-chromogen mixture was applied to the cells and incubated for 10 minutes. Slides were washed three times using fresh PBS. The slides were examined under a microscope (Olympus 1x70 fluorescent microscope).

Cell proliferation assay

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To determine if the clones grow at the same rate as un-transfected TC-1 cells, cell proliferation was checked with the MTS assay every 24 hours for 5 days. On day one, 4000 cells of each clone were plated on 12-well-plates in triplicates. At each time point, 3 wells of each clone were tested with MTS. The test was performed twice to obtain 6 replicates. Clones were compared using a one-way ANOVA with Turkey's post-test.

NK cell activation

Human NK92 cells and the cells of each clone at a ratio of 10:1 were plated on 96-well plate in 200 µl NK media and incubated for 48 hours. After incubation 100 µl of supernatant was collected from each well and analyzed for the presence of IFN-γ using BD OptiEIA human IFN-γ ELISA kit II following manufacturer's directions. The rest of the media as well as NK92 cells were removed by PBS washing. The proliferation of the remaining tumor cells was determined using Promega's CellTiter 96ueous nonRadioactive cell proliferation assay following the manufacturer's instructions. The data were analyzed using a one-way ANOVA with Turkey's post-test.

Apoptosis assay

One million cells of each clone were first plated on a 6-well-plate and incubated for 24 hours. At this point one million NK92 cells were added to the tumor cells and incubated for 2 hours. After removing NK cells, apoptosis of the tumor cells was determined by a caspase 3 assay kit (abcam) following the manufacturer's instruction.

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Results

<u>Fusion gene construction</u>

(a) Plasmid pcDNA3.1 (+) mIL-12/Fas$_{TI}$/Zeo

Mouse IL-12 cDNA was amplified from pORF-mIL-12 by PCR and cloned into mammalian expressing vector pcDNA3.1(+)/zeo with enzyme sites of HindIII and BamHI. The transmembrane/intracellular domains of mouse Fas was amplified from a previous plasmid made in our lab: pcDNA3.1 (+) MULT1E/Fas$_{TI}$/Zeo and cloned into the above vector with enzyme sites of BamHI and EcoRI. To allow an overall open reading frame and functional protein folding , the mIL-12 stop codon was removed and replaced with a (GGGS) 3' linker sequence The fusion gene sequence of the resulting plasmid pcDNA3.1 (+) mIL-12/Fas$_{TI}$/Zeo (Figure 2a) was confirmed by DNA sequencing.

(b) Plasmid pcDNA3.1 (+) mIL-12/Zeo

The IL-12 cDNA sequence without stop codon was amplified from pORF-mIL-12 and cloned into vector pDisplay with enzyme sites of XmaI and SacII In order to make the expression vector zeocin resistant, restriction enzymes HindIII and XhoI were used to cut plasmid pDisplay (+) mIL-12 and the fragment containing mIL-12 sequence together with the cell membrane anchoring sequence was retrieved and inserted into plasmid pcDNA3.1/Zeo. The sequence of the resulting plasmid pcDNA3.1 (+)/IL-12 (Figure 2b) was confirmed by DNA sequencing.

<u>Stable clone establishment</u>

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TC-1 lung carcinoma cells were transfected with pcDNA3.1 (+) mIL-12/Fas_{TI}/Zeo or pcDNA3.1 (+) mIL-12/ Zeo. Ten zeocin resistant clones were selected for each transfection, and the transgene expression was analyzed by RT-PCR. All ten clones are positive for fusion protein IL12/Fas_{TI} (Figure 3a) or cytokine IL12 (Figure 3b). The clones were then examined for protein expressing by IL-12 immunohistochemistry (IHC). IHC results demonstrated that fusion clones of IF-9, IF-10 and IL-12 clone IL12/8 expressed relatively high levels of the fusion protein or IL-12 on cell surface compared to control TC-1 cells (Figure 4).

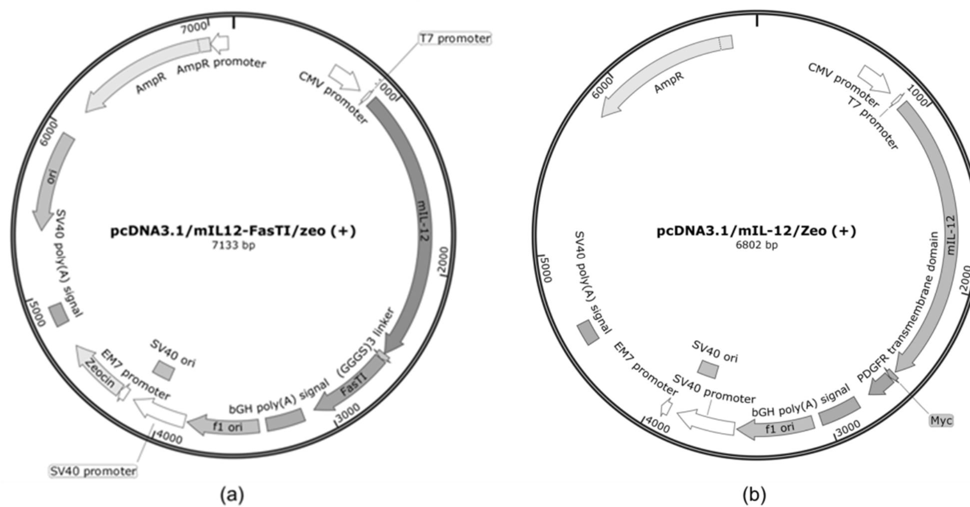

Figure2. Fusion gene constructs (a) Plasmid pcDNA3.1 (+) mIL-12/FasTI/Zeo. (b) Plasmid pcDNA3.1 (+) mIL-12/Zeo (containing PDGFR transmembrane domain)

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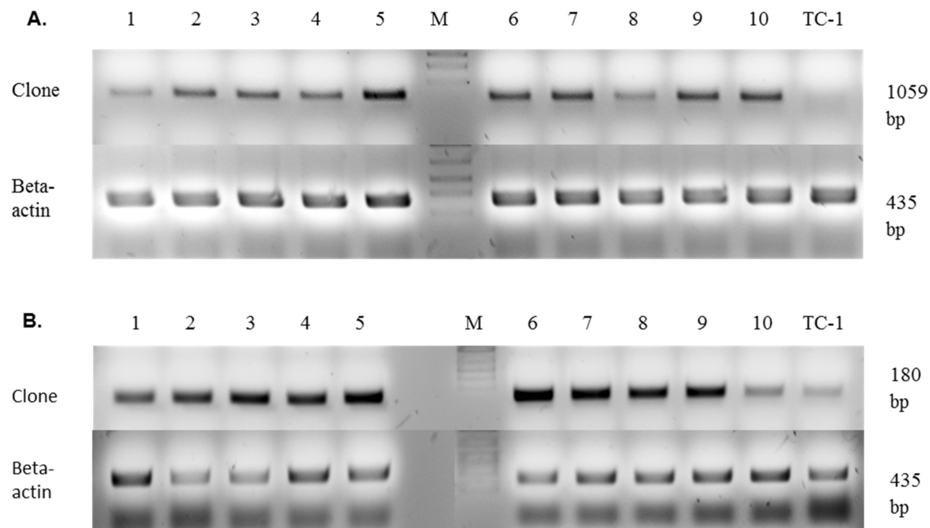

Figure 3. RT-PCR confirmation of gene expression in selected TC-1 clones. Total RNAs were isolated from the zeocin resistant clones and TC-1 cells and a two-step RT-PCR was performed using Phusion RT-PCR kit (Thermo Scientific).(a) Detection of fusion gene mIL-12/FasTI expression; (b) detection of IL-12 expression. House-keeping gene β-actin expression was also included as controls. Fusion gene clones of IL-12/FasTI/3, IL-12/FasTI/9 and IL-12/FasTI10 and IL-12 control clone IL-12/8 were selected for further studies.

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NK cell activation

It is critical to know whether fusion protein IL12/Fas_{TI} can effectively activate NK cells. Cells from TC-1 or clones of IF-9, IF-10 and IL12/8 were co-cultured with human NK92 cells. After the co-culture, the INF-γ production in the supernatant was detected by human IFN-γ ELISA analysis and the cytotoxicity of tumor cells was analyzed using Promega's CellTiter 96ueous nonRadioactive cell proliferation assay. The production of INF-γ was significantly increased by NK cells co-cultured with clone IF-9, IF-10 and IL12/8 compared to those co-cultured with TC-1 parental cells ($p<0.001$, $p<0.001$ and $p<0.05$, respectively) (Figure 5). More importantly, fusion protein IL-12/Fas_{TI} significantly enhanced the cytotoxicity of NK cells (Figure 6). NK cells co-cultured with clone IF9 and IF10 killed significantly more tumor cells ($p<0.01$, $p<0.01$) when compared with NK cells co-cultured with control TC-1 cells. It is interesting to know that although NK cells co-cultured with clone IL12/8, which express only IL-12 on cell surface, killed significant tumor cells ($p<0.05$), the cytotoxicity is relatively lower than NK cells co-cultured with fusion protein expressing cells.

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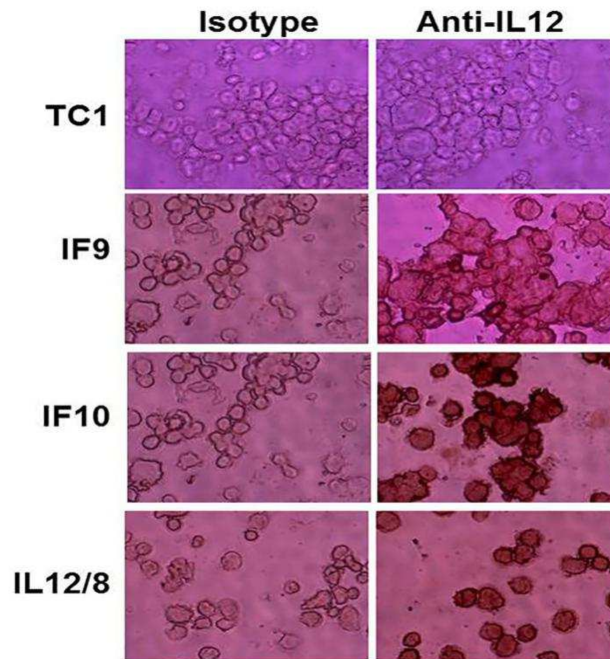

Figure 4. Fusion gene expression at protein level by Immunohistochemistry (IHC). Cells from clones and TC-1 cells were harvested and cytospun onto glass slides. After fixation, the cells were stained with anti-mouse IL-12 antibody or isotype control antibody followed by anti-mouse IgG secondary antibody conjugated with HRP. The cells were then treated with substrate DAB and observed under microscope.

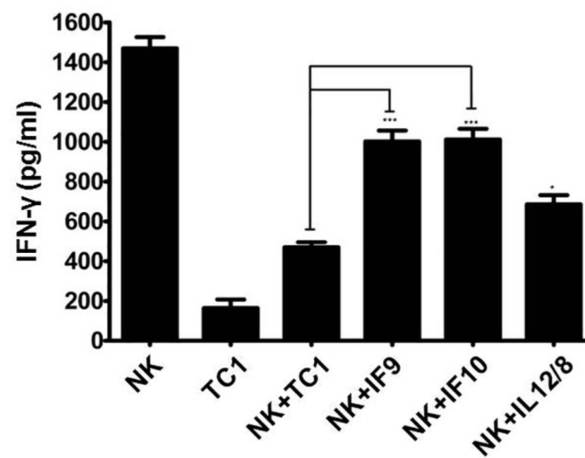

Figure 5. IFN-γ production by human NK92 cells. Cells of clones and TC-1 in triplicates were co-cultured with human NK 92 cells for NK cell activation and the supernatants were collected. IFN-γ activity in the supernatants was detected using Human IFN- γ ELISA Kit. The statistical analyses were conducted between the controls (NK+TC1) and IL-12 expressing control and IL12-FasTI expressing clones using one-way analysis of variance (ANOVA) with Tukey's post test. (*p<0.05; ***p<0.001)

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Fusion protein IL12/FasTI induces apoptosis of tumor cells

To confirm the hypothesis that when bound to IL-12 receptor, fusion protein IL-12/Fas_{TI} can also send death signals through its Fas_{TI} portion into the tumor cells, TC-1 cells and clones IF9, IF10 and IL12/8 were co-cultured with NK cells to induce apoptosis. Caspase 3 assay was performed to analyze the apoptosis in tumor cells. After co-culture, the caspase 3 activities of clone IF9 and IF10 were significantly higher than un-induced controls ($p<0.01$ and $p<0.001$, respectively). As expected, TC-1 and clone IL12/8 showed no significant difference with or without NK cell co-culture (Figure 7).

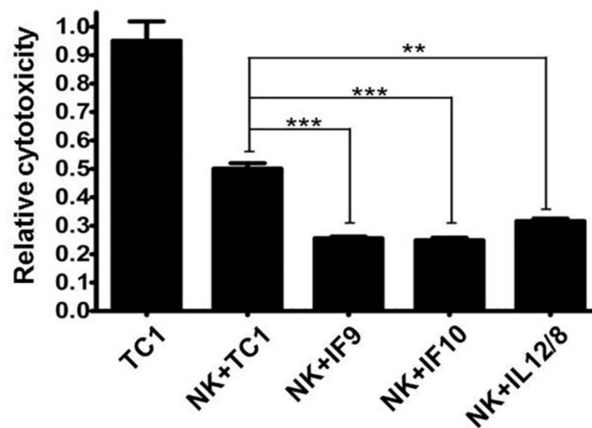

Figure 6. Cytotoxicity of NK cells. Cells of clones and TC-1 in triplicates were co-cultured with human NK 92 cells for NK cell activation. After co-culture, NK cells were removed and remaining tumor cells' proliferation was measured with Promega's CellTiter 96ueous nonRadioactive cell proliferation assay kit. The statistical analyses were conducted between the controls (NK+TC1) and IL-12 expressing control and IL12-FasTI expressing clones using one-way analysis of variance (ANOVA) with the Tukey's post test (**$p<0.01$, ***$p<0.001$)

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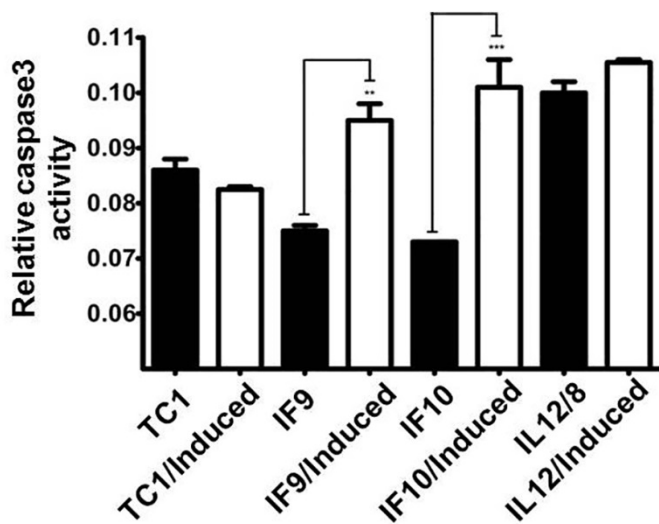

Figure 7. Induction of apoptosis in tumor cells. A total of 1x106 cells of TC-1 and clones IF9, IF10 and IL-12/8 in triplicates were co-cultured with NK92 cells for 2 hours. After the removal of NK cells, the caspase 3 activities in the remaining tumor cells were detected using a Caspase 3 Assay Kit (abcam). The statistical analyses were conducted between the corresponding un-induced and induced groups using one-way analysis of variance (ANOVA) with the Tukey's post test (**p<0.01, ***p<0.001)

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TC1 stable clones co-cultured with PBMC

TC-1 stable clones were co-cultured with human PBMCs on 96-well-plate for 24 hours. After co-cultural incubation, supernatant was harvested for human IFN-γ ELISA detection, while remaining cells of TC-1 stable clones were used for MTS cytotoxicity assay. It was shown that, after co-culture with PBMCs, TC1-IF clones produced significantly higher levels of IFN-γ ($p<0.001$, Figure 8). In contrary to our hypothesis, TC1-IL12 clones showed low production of IFN-γ as parental TC-1 cells. Fusion protein IL-12/Fas_{TI} significantly enhanced the cytotoxicity of PBMCs. PBMCs co-cultured with fusion clone and IL12 clone killed significantly more tumor cells ($p<0.01$, $p<0.01$) when compared with NK cells co-cultured with control TC-1 cells (Figure 9).

To further test the hypothesis that, when TC1-IF clones co-cultured PBMCs, fusion protein IL-12/Fas_{TI} can also send death signals through its FasTI portion into the tumor cells, TC-1 cells and clones IF-10 and IL12/8 were co-cultured with PBMCs to induce apoptosis. Caspase 3 assay and Annexin V apoptosis detection were performed to analyze the apoptosis activity (Figure 10). After co-culture, the caspase 3 activities of fusion clone were much higher than un-induced controls ($p<0.05$ and $p<0.001$, respectively). It is interesting to find out that IL12 clone also showed increased caspase 3 activity after co-culture with PBMCs, which implies that apoptosis was induced by both Fas-mediated apoptosis signaling as well as other mechanisms. Meanwhile, Annexin V and Propidium Iodide (PI) staining were performed to identify dead/apoptotic/alive cells after co-culture with PBMCs. Cells undergoing apoptosis displayed brighter green staining, while dead cells red and alive cells faint green. The result indicates that TC1-IF10 exhibited much

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higher apoptosis level comparing to TC1-IL12 and TC1 cells co-cultured with PBMCs, which agrees with the caspase 3 activity.

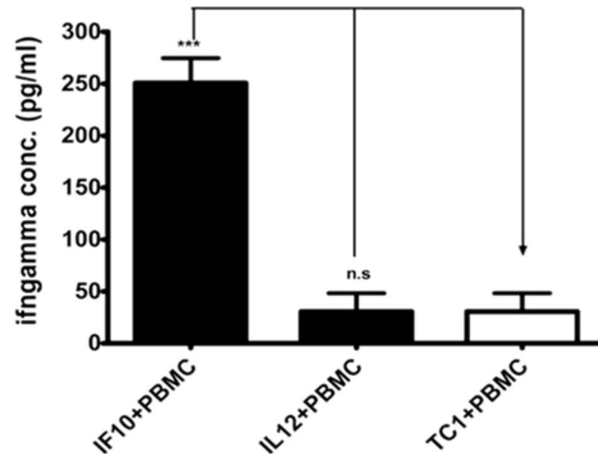

Figure 8. IFN-γ production by human PBMCs. Cells of clones and TC-1 in triplicates were co-cultured with human PBMCs for IFN-γ production detection. IFN-γ ac tivity in the supernatants was detected using Human IFN-γ ELISA Kit. The statistical analyses were conducted between the controls (PBMC+TC1) and IL-12 expressing control and IL12-FasTI expressing clones using one-way analysis of variance (ANOVA) with Tukey's post test. (***p<0.001)

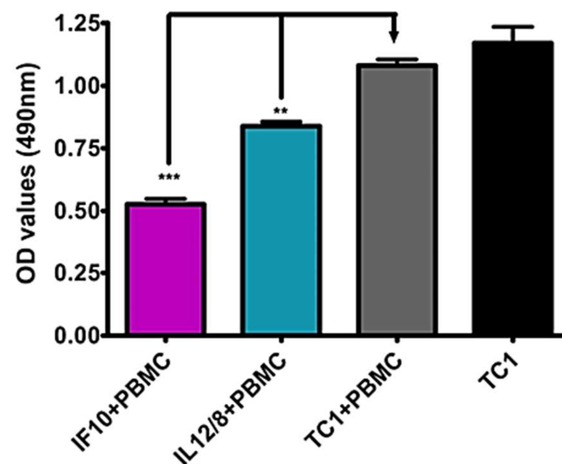

Figure 9. Overall cytotoxicity of human PBMCs. Stable clones and TC-1cells in triplicates were co-cultured with human PBMCs for anti-tumor cytotoxicity detection. After co-culture, PBMCs were removed and remaining tumor cells' proliferation was measured with Promega's CellTiter 96ueous nonRadioactive cell proliferation assay kit. The statistical analyses were conducted between the controls (PBMCs+TC1) and IL-12 and IL12-FasTI expressing clones using one-way analysis of variance (ANOVA) with the Tukey's post test (**p<0.01, ***p<0.001)

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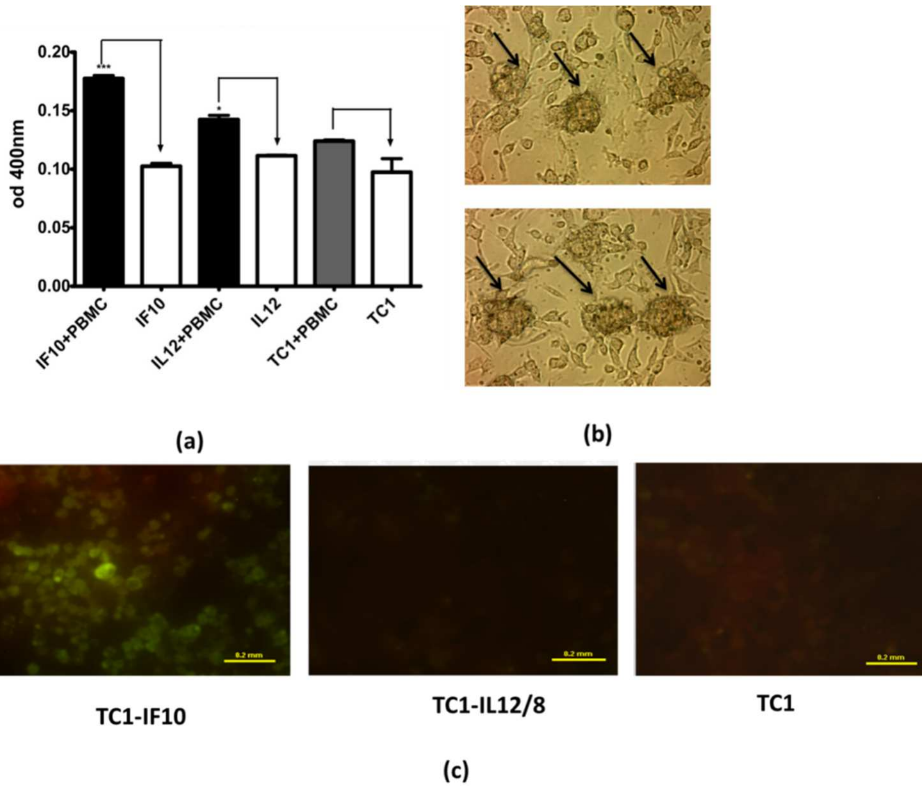

Figure 10. Induction of apoptosis in tumor cells by human PBMCs. **(a)** A total of $1x10^6$ cells of TC-1 and fusion clone IF10 and IL-12/8 in triplicates were co-cultured with PBMCs for 4 hours. After the removal of PBMCs, the caspase 3 activities in the remaining tumor cells were detected using a Caspase 3 Assay Kit (abcam). **(b)** Cluster of apoptotic cells were observed under the microscope four hours after co-culture with PBMCs. **(c)** Annexin V and PI staining were performed to analyze apoptosis level. Alive cells show faint green, apoptotic cells bright green and dead cells red staining. The images were taken using Olympus fluorescent microscopy.

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Discussion

Different strategies using NK cells for anti-tumor immunotherapy such as in vivo cytokine therapy to expand and activate NK cells against tumor cells have been used [TM Law et al. 1995; NJ Meropol et al. 1998]. However, a majority of these studies failed to demonstrate significant clinical benefit. As tumors have developed multiple mechanisms to subvert and suppress immune responses by deregulating cell surface expression of Fas and Fas ligand resulting in reduced sensitivity of tumor cells to Fas-mediated apoptosis. This resistance to Fas-mediated apoptosis protects tumor cells from killing by infiltrating antitumor NK cells and T cells [U Elsasser-Beile et al. 2003] . NK cells require activating receptors as well as activating cytokine, like IL-12, to function. The systemic infusion of IL-12 in high amounts is effective in activating an immune response against tumor cells but it is toxic to most patients. In this study, we introduced mouse IL-12, one of most important and potent NK cell activating cytokine, and the DD of Fas in the transmembrane and intracellular domain into one fusion protein IL12/FasTI. It is hypothesized that the development of this bi-functional chimeric protein can send an apoptosis signal to the tumor cells and at the same time activate NK cells and other immune cells.

The transcription of fusion protein in zeocin resistant clones was confirmed by RT-PCR (Figure 3) and the expression of the fusion protein was further characterized by cell surface immunohistochemistry (IHC, Figure 4). In the RT-PCR experiment, pair of primers were designed to cover the entire IL-12 fragment and partial upstream fragment of Fas_{TI}; thus only clones expressing fusion protein should show a band. In the IHC

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analysis, when anti-mouse IL12 antibody was used, clone IF-9, IF-10 and IL-12/8 showed positive, whereas TC-1 cells were negative (Figure 4).

Increased production of IFN-γ is a key indicator of NK cell activation. To confirm the idea that fusion protein IL12/FasTI activates immune cells that express IL-12R, human NK92 cells were co-incubated with cells of TC-1 and clones IF-9, IF-10 and IL-12/8 and IFN-γ expression was detected by human IFN-γ ELISA. The IFN-γ expression by NK cells co-cultured with cells of clones IF-9, IF-10 and IL-12/8 increased significantly compared to those of NK cells co-cultured with cells of TC-1 (Figure 5). Interestingly, NK cells without co-culture produced the most IFN-γ. This might be due to the fact that tumor cells express ligands for inhibitory receptors on NK cells. [18]. Therefore, the logic comparison should be between NK cells co-cultured with TC-1 and clones expressing the fusion genes or IL-12. More importantly, the cytotoxicity assay further demonstrated that fusion protein clones IF-9 and IF-10 are more effective in inducing NK mediated cell killing than IL-12/8 clone expressing membrane anchored IL-12. This may be due to the additive effect of FasTI induced apoptosis. (Figure 6).

Another key feature of the designed fusion protein is to send apoptosis signals into the cells expressing the protein after binding to its ligand. Although we do not have direct evidence supporting that the binding of IL-12 to IL-12R on NK cells can trigger FasTI to form DISC inside tumor cells, a clear apoptosis signal is send to targeted tumor cells as indicated by the increased caspase-3 activity after co-culture of the clones with NK cells (Figure 7). This also agrees with the results of cytotoxicity study (Figure 6).

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To better simulate the *in vivo* immune environment, PBMCs not NK cells alone were used to co-culture with TC-1 stable clones to evaluate their anti-tumor efficacy. Interestingly, when co-cultured with PBMCs, IF10 fusion clone also showed significantly enhanced IFN-γ production, apoptosis level as well as overall cytotoxicity against target tumor cells, comparing to TC-1 cells co-cultured with PBMCs. Noted that when co-cultured with PBMCs, IL-12/8 clone displayed higher caspase 3 activity than co-culture with NK92 alone (Figure 7, 10). Because PBMCs consist of T cells, B cells, NK cells and monocytes, it is implied that IL-12 might be engaged with other lymphocytes than NK cells to induce apoptosis and other anti-tumor mechanisms.

In conclusion, a bi-functional chimeric protein containing the extracellular domain of mouse IL-12 and transmembrane and intracellular domains of Fas is created. In vitro studies demonstrate that the fusion protein can activate NK cells for anti-tumor cytotoxicity and induce apoptosis in tumor cells. When combined with an effective tumor specific gene delivery vehicle, the bi-functional fusion gene represents a novel approach for cancer immunotherapy.

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CHAPTER FOUR

LENTIVIRAL DELIVERY OF NOVEL FUSION PROTEIN INTERLEUKIN-12/FASTI FOR CANCER IMMUNEGENE THERAPY

Abstract

Many of the cytokine-based cancer immunotherapies are hindered by the devastating side effects of systemic delivery of the cytokines. To address this problem, we previously described a novel approach to locally achieve high doses of IL-12 in tumors and demonstrated that bi-functional fusion protein mIL-12/Fas_{TI} expressed by stable clones of TC-1 cells efficiently suppressed tumor proliferation by activating NK cell and other cytolytic killer cells and sending apoptotic signals into tumor cells [Yang et al. 2016]. In the present study, we employed a lentiviral vector-based gene delivery system to deliver this fusion construct directly into tumor cells. We show that lentiviral vector efficiently delivers the fusion constructs into Hela cells in vitro as assayed by RT-PCR and immunohistochemistry (IHC). We also confirm that fusion protein mIL-12/Fas_{TI} delivered by the viral vector significantly enhanced killer cell activation, increased caspase-3 activity and decreased tumor growth *in vitro*. This study offers a further step for fusion protein cancer therapy for cancer patients.

Key words: lentiviral vector, IL-12, Fas, NK cell, apoptosis

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Introduction

Natural killer (NK) cells are specialized lymphocytes capable of targeting virus-infected cells and malignant cells such as tumor cells. NK cell activation requires engagement of pro-inflammatory cytokines, particularly such as interleukin-12 (IL-12). Fas (CD95/APO-1) is a cell surface death receptor of tumor necrosis factor (TNF) receptor superfamily. Binding of Fas ligand to Fas recruits Fas-associated death domain (FADD) and induces apoptosis.

In our previous study, we have demonstrated that mouse IL-12/Fas_{TI}, a bi-functional fusion protein containing mouse IL-12 and Fas transmembrane/ intracellular domain (Fas_{TI}), can simultaneously enhance NK cell activity and induce apoptosis of tumor cells in a tumor microenvironment, resulting in effective tumor cell elimination in vitro(Yang *et al.*, 2016). To further confirm the anti-tumor efficacy of fusion protein IL-12/Fas_{TI} *in vivo*, high-efficient gene delivery method is in demand. Lentiviruses are a subgroup of the retrovirus family, which have been developed mostly from simian immunodeficiency virus (SIV) and human immunodeficiency virus type I (HIV-1). The design of lentivirus-based expression vectors allows high-level expression of recombinant fusion proteins in diving and non-diving mammalian cells. In the present study, lentiviral vectors, containing the cDNA sequence of fusion gene *mIL12/*Fas_{TI} (pLenti7.3/ mIL12/Fas_{TI}) and the control gene (pLenti7.3/IL12, pLenti7.3/Fas), were established through advanced molecular cloning. This pLenti7.3/V5-TOPO lentiviral expression vector contains two new elements to yield cell-specific, high performance results; the WPRE (Woodchuck Posttranscriptional Regulatory Element) and cPPT

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(Polypurine Tract) together can produce at least a four-fold increase in viral titer (Follenzi, et al. 2000; Zuffery et al. 1999). The titer of each viral clone was determined by transducing human Hela cervic carcinoma cells. The transient protein expression of IL-12/Fas_{TI}, IL-12 and Fas via lenti-viral transduction was confirmed by both reverse-transcription PCR (RT-PCR) and immunohistochemistry (IHC). It was also demonstrated that the expression of IL-12/Fas_{TI} enhanced apoptosis levels, NK cell activity as well as overall cytotoxicity against tumor cells, comparing to IL-12 and Fas control. Combined with high-efficient lentiviral expression system, our fusion protein strategies might serve as one potential option for cancer immunogene therapy in the future.

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Materials and Methods

Cells

293 cells (ATCC No. CRL-1573) were cultured in Eagle's Minimum Essential Medium containing 10% fetal bovine serum and 100μg/ml gentamicin at 37℃ with 5% CO_2.

Human 293FT (Thermo Fisher Scientific Cat# R700-07) were cultured in D-MEM medium containing 10% FBS supplemented with 0.1 mM MEM Non-Essential Amino Acids, 1 mM sodium pyruvate, 2 mM L-glutamine and 500μg/ml Geneticin as "selective antibiotics") at 37℃ with 5% CO_2.

Human peripheral blood mononuclear cells (PBMCs) were isolated from blood samples. PBMCs were cultured in RPMI1640 media containing 20% fetal bovine serum, 100 μg/ml gentamicin and 100 IU IL-2 at 37℃ with 5% CO_2.

Human cervical carcinoma Hela cells were cultured in high-glucose DMEM containing 10% fetal bovine serum and 100μg/ml gentamicin at 37℃ with 5% CO_2.

Construction of lentiviral vectors

Mouse *IL-12/Fas$_{TI}$* cDNA sequence was cloned from pcDNA3.1/IL-12/Fas$_{TI}$/Zeo (+) vector from previous study using ACCATGGGTCAATCACGCTAC as 5' primer and CTCCAGACATTGTCCTTCATTTTC as 3' primer. The optimal sequences for translation initiation (Kozak sequences) were amplified in the DNA. To enable TA cloning with Plenti7.3/V5-TOPO vector, *Taq* DNA polymerase was used for PCR to

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generate extruding "A" at both ends of PCR product. 4µl purified PCR product of IL-12/Fas_{TI}, 1µl Salt solution and 1µl pLenti7.3/TOPO vector (Invitrogen, USA) were then mixed and incubated at room temperature for 5 minutes for TOPO cloning reaction. Six ampicillin-resistant colonies from transformation were picked, and plasmid DNA for each colony was isolated using a QIAprep Spin miniprep kit (Qiagen). The plasmid was analyzed by restriction enzyme digest and DNA Sanger sequencing to confirm the presence and orientation of insert as well as the integrity of the vector.

Mouse IL-12 cDNA sequence was cloned from pcDNA3.1/IL-12/Zeo (+) from previous study using ACCATGGGTCAATCACGCTAC as 5' primer and CGTGGCTTCTTCTGCCAAAGCATG as 3' primer. Mouse Fas cDNA sequence was cloned from pCMV-mFAS-His purchased from Sino Biological Inc., using ACCATGGTGTGGATCTGG as 5' primer and CTCCAGACATTGTCCTTCATTTTC as 3' primer. PLenti7.3/IL-12 and pLenti7.3/Fas were constructed by the same methodology following manufacturer's instructions. PLenti7.3/IL-12 and pLenti7.3/Fas sequences were analyzed by DNA Sanger sequencing to confirm the presence and orientation of insert as well as the integrity of the vector.

Production of lentiviral particles in 293FT cells

293FT cells were co-transfected with pLenti7.3/mIL-12/Fas_{TI}, pLenti7.3/mIL-12, or pLenti7.3/mFas, respectively, as well as lentiviral packing mix (containing three packing plasmids, pLP1, pLP2 and pLP/VSVG) using Lipofectamine 2000 (Invitrogen) as directed by the manufacturer's instructions. Virus-containing supernatant of each pLenti expression construct was harvested 48-72 hours post-transfection directed by

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manufacturer's instruction. Meanwhile, pLenti7.3/V5-GW/LacZ was used as a control in co-transfection to optimize expression conditions. Before proceeding to transduction and expression experiments, lentiviral stock of each construct was concentrated by using Lenti-X concentrator (ClonTech) and the viral titer was determined for further analyses. Lentiviral titration was determined by fluorescence-based cytometry of GFP positive cells following manufacturer's instruction.

RT-PCR

Total RNA was extracted from each Lenti-clone using an RNeasy Plus from Qiagen following the manufacturer's directions. cDNAs were produced from 2μg of the total RNA using the QuantiTect RT kit from Qiagen following the manufacturer's directions. Five microliters of the cDNA product were added to a PCR reaction containing primers amplifying a 1059-bp portion of mIL-12/Fas_{TI} fusion gene sequence containing 522-bp of the mIL-12, the $(GGGS)_3$' linker sequence (GGTGGTGGTTCTGGTGGTGGTTCTGGTGGTGGTTCT) , and the entire 495-bp of FasTI. A Phusion High-Fidelity DNA polymerase kit was used with a 5' primer GCAGTGACATGTGGAATGGC and a 3' primer CGGAATTCTCACTC CAGACATTGTCCTTCATTTTC. Similar reaction was also carried out to amplify portion of mIL-12 sequence using 5' primer GGAAGCACGGCAGCAGAATA and 3' primer AACTTGAGGGAGAAGT AGGAATGG to amplify a 180-bp fragment of mIL-12. To amplify 984-bp of Fas sequence, a 5' primer ACCATGGTGTGGATCTGG and a 3' primer CTCCAGACATTGTCCTTCATTTTC were used.All RT-PCR reactions

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included the β-actin housekeeping gene as loading control using a 5'primer ATGGGTCAGAAGGATTCCTATGTG and a 3' primer CTTCATGAGGTAGTCAGTCAGGTC amplifying a 488-bp fragment.

Immunohistochemistry (IHC)

Glass slides having cells of virally transduced clones were prepared using cytospin technology. 100,000 cells in 1 ml PBS were transferred into assembled cytospin cassettes and centrifuged in the CytoTek centrifuge at 2,000 rpm for 10 minutes at room temperature. After centrifuge the cells on the slides were fixed with 4% para-formaldehyde solution for 10 minutes. The cells were then stained using a cell staining kit (R& D Systems) following manufacturer's directions. Rat IgG_{2a} antibody was used as isotype control (R&D Systems, clone #54447); anti-mouse IL-12/p35 antibody and anti-Fas antibody were used for IL-12 staining and Fas staining (R&D Systems, clone #45806). Both primary antibodies were diluted as 1:100 by sterile PBS. Primary antibodies were incubated with the cells for an hour at room temperature. Secondary antibody conjugated with HSS-HRP was then incubated with the cells for 30 minutes at room temperature. DAB substrate-chromogen mixture was applied to the cells and incubated for 10 minutes. Slides were washed three times for 2 minutes using fresh PBS between steps. After the final incubation, the slides were rinsed with distilled water for 5 minutes, and then observed with a microscope (Olympus 1x70 fluorescent microscope).

Coculture of human NK92 cells with lentiviral transduced Hela cells

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Human NK cells (NK92) were plated with Hela cells, or lentiviral transduced Hela cells expressing IL-12/Fas_{TI}, IL-12 or Fas on 96-well plate at a ratio of 1:1 in 200 µl NK media for 48 hours. After co-cultural incubation, 100 µl of supernatant was removed for analysis of IFN-γ production using human IFN-γ ELISA reagent kit (Thermo Fisher Scientific) following manufacturer's direction. The remaining media was removed, and PBMC suspension cells were carefully removed. The number of remaining tumor cells were determined using CellTiter 96 AQueous non-radioactive cell proliferation assay (Promega) following manufacturer's directions. The results were analyzed using a one way ANOVA with Tukey's post-test.

Coculture of human PBMCs with lentiviral transduced Hela cells

Human PBMCs were plated with Hela cells, or lentiviral transduced Hela cells expressing IL-12/Fas_{TI}, IL-12 or Fas on 96-well plate at a ratio of 5:1 in 200 µl NK media for 48 hours. After co-cultural incubation, 100 µl of supernatant was removed for analysis of IFN-γ production using human IFN-γ ELISA reagent kit (Thermo Fisher Scientific) following manufacturer's direction. The remaining media was removed, and PBMC suspension cells were carefully removed. The number of remaining tumor cells were determined using CellTiter 96 AQueous non-radioactive cell proliferation assay (Promega) following manufacturer's directions. The results were analyzed using a one way ANOVA with Tukey's post-test.

Tumor cell apoptosis

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One million cells of Hela cells or lentiviral transduced Hela cells expressing IL-12/Fas_{TI}, IL12 or Fas were first plated on a 6-well-plate and incubated for 24 hour. At this point one million PBMCs were added to all the cells and incubated overnight for coculture. After removing suspension PBMCs, apoptosis of the tumor cells was determined by a caspase 3 assay kit (abcam) following the manufacturer's instruction.

Statistical analysis

GraphPad software package was used to plot graphs and run statistical analysis. The statistical significance was represented as *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results

Lentiviral expression vector constructs

Mouse *IL-12/Fas_{TI}* cDNA sequence was amplified from pcDNA3.1/IL-12/Fas_{TI}/Zeo (+) by PCR into lentiviral expression vector PLenti7.3/V5_TOPO using TA cloning. Control mouse *IL-12* and *Fas* sequence were cloned into PLenti7.3/V5_TOPO vector using TA cloning. The resulting Lentiviral constructs were confirmed by restriction enzyme digest and DNA Sanger sequencing for orientation of ligation and integrity of entire plasmid (Figure 11).

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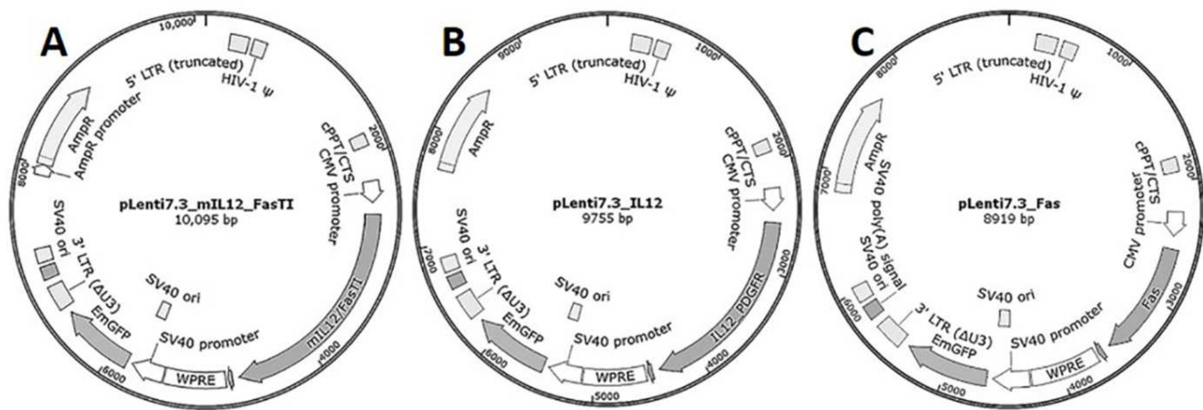

<u>**Figure 11.**</u> Constructs of lentivirus-based gene expression vectors. (a) Construct of Plenti7.3/mIL12_FasTI/V5-TOPO; (b) Construct of Plenti7.3/mIL12/V5-TOPO; (c) Construct of Plenti7.3/mFas/V5-TOPO.

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Lentiviral titer determination

293FT cells were co-transfected with Lent-IF, Lent-IL12, and Lent-Fas, respectively, and lentiviral packing mix using Lipofectamine 2000. Virus-containing supernatant of each Lent-construct was harvested 48-72 hours post-transfection. The lentiviral particles were first concentrated using Lenti-XTM Concentrator (Clontech). Concentrated viral particles were then assayed to determine their viral titers. Emerald Green fluorescent protein (EmGFP) gene carried with the lentiviral vectors allows convenient determination of the lentiviral titers. 293 cells were transduced with Lent-IF, Lent-IL12, Lent-Fas and Lent-LacZ, respectively, for 48 hours, and virally transduced cells as well as un-transduced 293 cells were collected to determine percentage of EmGFP positive cells by Tali™ image-based cytometry (Invitrogen). The percentages of fluorescent cells in Lent-IF, Lent-IL12, Lent-Fas and Lent-LacZ transductions are 58.1%, 47.2%, 45.9 and 60.2%. The titer was then calculated following the equation: titer = $\{(F \times Cn) / V\} \times DF$ (F: The frequency of GFP-positive cells determined by cytometry; Cn: The total number of target cells infected; V: The volume of the inoculum; DF: The virus dilution factor). The titers are between 1.03 to 1.35×10^7 /ml.

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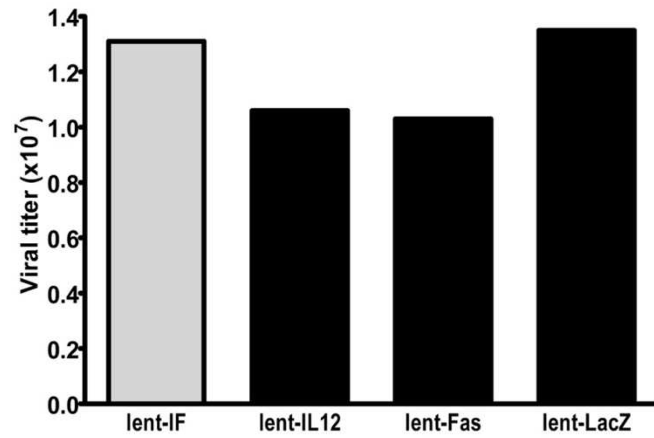

Figure 12. Lentiviral titer determination. Each virally transduced 293-Lent clones (Lent-IF/Lent-IL12/Lent-Fas/Lent-LacZ) were collected for EmGFP-based cytometry analysis. Viral titer of each Lent-clone was calculated based on cytometry reading and represented.

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Gene expression via lentiviral delivery system

Human cervical cancer Hela cells were transduced with Lent-IF, Lent-IL12, or Lent-Fas and RT-PCR and IHC were performed to determine the gene expressions. RT-PCR shows that after lentiviral transduction, all the three constructs, Lent-IF, Lent-IL12, and Lent-Fas are transcribed correctively (Fig.13). IHC results demonstrated that IL12/Fas and IL-12 proteins are expressed on the cell surface (Fig.14). Fluorescent microscopic analysis showed that control Fas protein was also expressed on the cell surface. (data not shown)

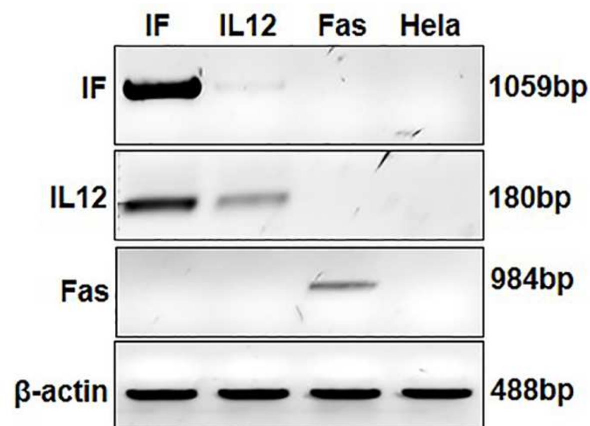

Figure 13. RT-PCR Confirmation of gene expression in lentiviral transduced cells. Total RNAs were isolated from Lent-clones and un-transduced Hela cells and a two-step RT-PCR was performed using Phusion RT-PCR kit (Thermo Scientific) House-keeping gene β-actin expression was also included as controls.

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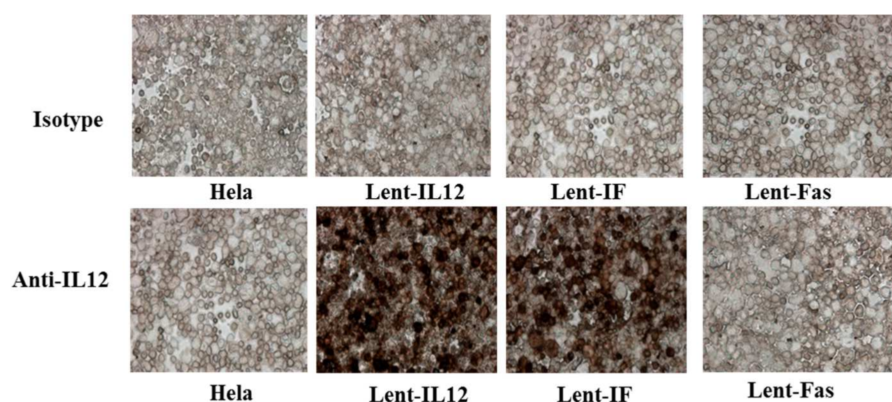

Figure 14. IHC staining of lentiviral transduced cells. Cells from Lent-clones and Hela cells were harvested and cytospun onto glass slides. After fixation, the cells were stained with anti-mouse IL-12 antibody or isotype control antibody followed by anti-mouse IgG secondary antibody conjugated with HRP. The cells were then treated with substrate DAB and observed under microscope.

Lentivirally transduced cells cocultured with human NK cells

To demonstrate if the bi-functional fusion protein IL-12/Fas_{TI} delivered by the lentiviral system can activate killer cells, lentivirally transduced Hela cells (Lent-IF/IL12/Fas) as well as Hela cells alone were cocultured with human NK92 cells for 48 hrs. One hundred microliter supernatant was collected from each well and screened by human IFN-γ ELISA. After coculture treatment, remaining transduced/un-transduced cells were quantified by MTS cytotoxicity assay. The IFN-γ ELISA results indicate that IL-2 dependent NK92 cell culture will simultaneously produce IFN-γ. When cocultured with Lent-IF and Lent-IL12 virally transduced Hela cells, IFN-γ production was obviously enhanced (not statistically significant) (Figure 15).

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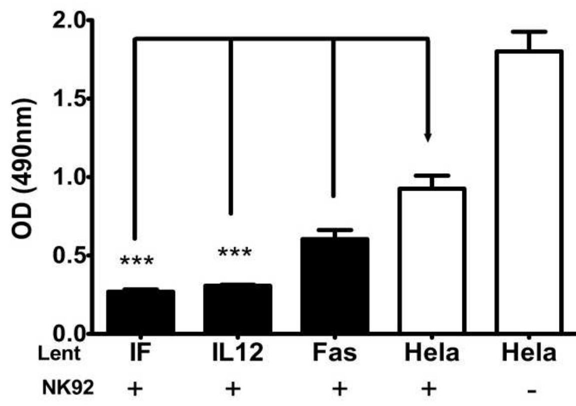
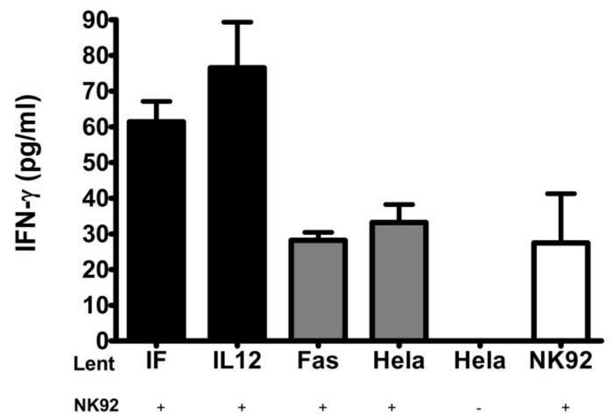


(a)



(b)

Figure 15. Activation of human NK92 cells when cocultured with lentiviral transduced cells. (a) Cytotoxicity of NK cells. Cells of each lent-clone and Hela in triplicates were co-cultured with NK92 cells. After co-culture, NK92 cells were removed and remaining tumor cells' proliferation was measured with Promega's CellTiter 96ueous nonRadioactive cell proliferation assay kit. (b)Human IFN-γ ELISA. Cells of Lent-clones and Hela cells in triplicates were co-cultured with human NK92 cells and the supernatants were collected. IFN-γ activity in the supernatants was detected using Human IFN-γ ELISA Kit. The statistical analyses were conducted between the control (Hela+NK92) and each virally transduced lent-clone using one-way analysis of variance (ANOVA) with the Tukey's post test (***$p<0.001$)

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Human PBMCs activation

In order to further test the activation of anti-tumor immune responses in a more therapeutic setting, human PBMCs were isolated and cocultured with Hela cells transduced with Lent-IF, Lent-IL12, or Lent-Fas for 48 hrs. One hundred microliter supernatant was collected from each well and screened for IFN-γ production by ELISA. PBMCs cocultured with Hela cells transduced with Lent-IF and Lent-IL12 showed significantly increased production of IFN-γ comparing to the controls (Fig.16a). While the IFN-γ production by PBMCs cocultured with Hela cells transduced with Lent-IF is not statistically higher than that of PBMCs cocultured with Hela cells transduced with Lent-IL12, the enhanced trend is clear. Interestingly, PBMCs cocultured with Hela cells transduced with Lent-Fas produced significantly higher IFN-γ than control Hela cells. The cytotoxicity of tumor cells after co-culturing with PBMCs were measured with MTS assay. PBMCs co-cultured with Hela cells transduced with Lent-IF and Lent-IL12 killed much more tumor cells than the controls (Fig.16). More importantly, Lent-IF is significantly more effective than Lent-IL12.

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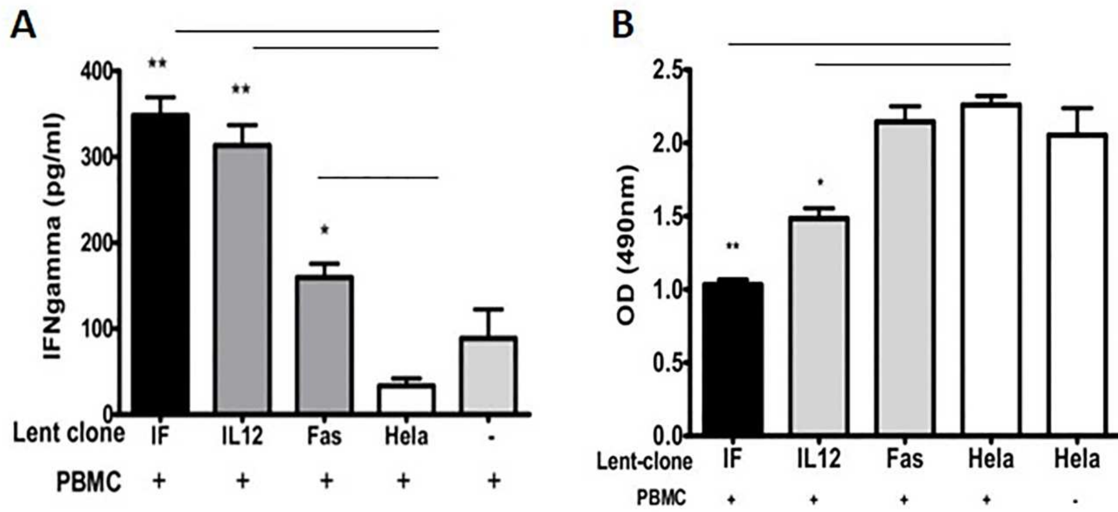

Figure 16. Activation of human PBMCs when cocultured with lentiviral transduced cells . (a) IFN-γ ELISA. Cells of Lent-clones and Hela cells in triplicates were co-cultured with human PBMCs and the supernatants were collected. IFN-γ activity in the supernatants was detected using Human IFN-γ ELISA Kit. The statistical analyses were conducted between the control (Hela+PBMCs) and each Lent-clone using one-way analysis of variance (ANOVA) with Tukey's post test. (*p<0.05; **p<0.01) (b) Cytotoxicity of PBMCs. Cells of each lent-clone and Hela in triplicates were co-cultured with human PBMCs. After co-culture, PBMCs were removed and remaining tumor cells' proliferation was measured with Promega's CellTiter 96ueous nonRadioactive cell proliferation assay kit. The statistical analyses were conducted between the control (Hela+PBMCs) and each virally transduced lent-clone using one-way analysis of variance (ANOVA) with the Tukey's post test (*p<0.05; **p<0.01)

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Apoptosis induction

PBMCs were cocultured with Hela cells transduced with Lent-IF, Lent-IL12 or Lent-Fas for 24 hours to induce apoptosis within the target cells. Caspase 3 assay was performed to analyze the apoptosis in tumor cells. Lent-IF induced significantly higher caspase 3 activity in the transduced Hela cells than all the controls (Fig.16).

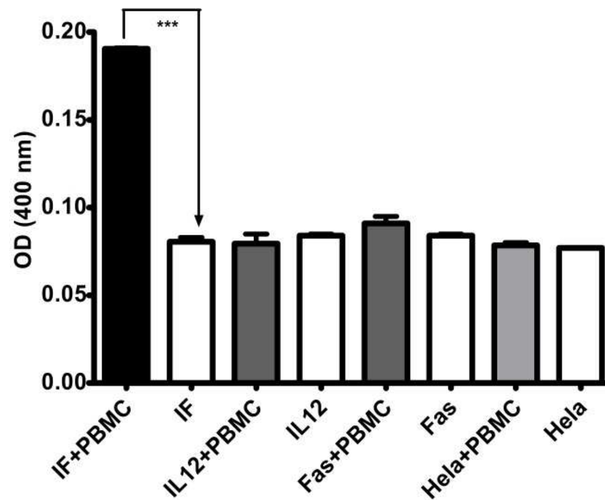

Figure 17. Induction of apoptosis in tumor cells via lentiviral transduced clones. A total of 2x105 cells of Hela cells and lent-clones in triplicates were co-cultured with PBMCs for 24 hours. After the removal of suspension PBMCs, the caspase 3 activities in the remaining tumor cells were detected using a Caspase 3 Assay Kit (abcam). Images of apoptotic cells were shown in the figure. The statistical analyses were conducted between the corresponding un-induced and induced groups using one-way analysis of variance (ANOVA) with the Tukey's post test (***p<0.001).

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Discussion

The role of the immune system in recognition and elimination of tumor cells has been well recognized. NK cells are one of the key players in anti-tumor immune response [Waldhauer I et al. 2008]. NK cells and other killer cells are key players in anti-tumor immune response. NK cell activation requires cytokine IL-12. In our previous study, we built a bi-functional fusion gene IL-12/Fas_{TI} containing mouse IL-12 sequence and Fas transmembrane and intracellular domains and demonstrated its designed activities *in vitro*.

To examine the bi-functional fusion protein's anti-tumor activity in a therapeutic setting, a lentivirus-based gene delivery system was utilized to deliver the fusion gene construct into human tumor cells. While the raw transfection supernatants contain relatively less viral particles, after concentration, a titer of over $1x10^7$/ml was achieved (Fig.12). After transduction, Hela cells functionally expressed the transduced genes (Fig.13 and Fig.14).

The major purpose of building the bi-functional fusion protein is to achieve local high level expression of IL-12 to activate NK cells and other killer cells and simultaneously send apoptotic signal to tumor cells. Therefore, human NK92 cell line was utilized for our co-culture model *in vitro*, which was cocultured with Hela cells that were virally transduced with Lent-IF, Lent-IL12 and/or Lent-Fas. The activation of NK cells was evaluated via human IFN-γ ELISA. Although our data obviously suggest that Lent-IF and Lent-IL12 enhanced IFN-γ production after coculture, the difference is not statistically significant comparing to control (Hela+NK92). Interestingly, we found that

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NK92 cell alone also displayed certain amount of IFN-γ production, implying that to some degree, IL-2 dependent NK92 cell culture could spontaneously produce IFN-γ.

To further test the biological functions of our fusion protein in a more therapeutic setting, human PBMCs isolated from donor blood were used to replace NK92 cells for our coculture model. Human PBMCs, which contain NK cells and other killer cells, were co-cultured with Hela cells transduced with Lent-IF, Lent-IL12 and/or Lent-Fas. The activation of PBMCs after co-culture was determined by the production of IFN-γ (Fig. 16a). It is not a surprise that PBMCs co-cultured with Hela cells transduced with Lent-IF and Lent-IL12 produced a similar amount of IFN-γ because the IL-12 portion of the fusion protein is identical to mouse IL-12. It is a surprise that PBMCs co-cultured with Hela cells transduced with Lent-Fas produced more IFN-γ than PBMCs co-cultured with Hela cells. Further studies are needed to find out if Fas signaling is involved in IFN- γ production via NK cells or other cells. Fusion protein IL-12/Fas_{TI} clearly demonstrated enhanced overall elimination against tumor cells comparing to IL12 control, while Fas control showed no effect on anti-tumor cytotoxicity. (Fig. 16b)

Apart from NK cell activation, the fusion gene design also involves Fas-induced apoptosis signaling in transmembrane and intracellular domains. The data demonstrated that strong apoptotic signals were sent into tumor cells that express the fusion protein IL12/Fas_{TI}, not cells that express IL-12 or Fas, when co-cultured with PBMCs (Fig. 17). The data also confirmed that mouse Fas is able to send apoptotic signals in human cells (Lamboley *et al.*, 2002).

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In conclusion, lentivirus-based gene expression system was successfully used to deliver fusion gene construct IL12/Fas_{TI} into tumor cells with potential efficiency, which provided a further step for fusion protein strategies in cancer immune/gene therapies. Furthermore, the fusion gene product IL-12/Fas_{TI} produced by lentiviral transduction effectively activated killers as indicated by the enhanced production IFN-γ and tumor cell cytotoxicity.

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CONLUSION

Although tumor immunosurveillance plays a vital role in controlling tumor progress and metastasis, a tumor inhibitory microenvironment in advanced tumors has disabled the immune destruction against malignant expansion. Considering the diverse escape mechanisms utilized by sneaky tumor cells, it is critical to revoke the immune system against tumor cells again by cell/gene therapy. In our study, fusion protein strategy was utilized to combine different anti-tumor effectors into one bifunctional chimeric protein. Our novel fusion protein consists of interleukin-12 (IL12) at upstream, as well as Fas transmembrane/intracellular domains at downstream of the construct. Once delivered to tumor cells, the fusion protein IL12/Fas_{TI} will be anchored on the tumor cell surface. Extracellular IL12 can direct to circulating natural killer (NK) cells by targeting and binding to IL12 receptor (IL12R). IL12/IL12R axis is hypothesized to stimulate NK cell activation in the tumor microenvironment, which will further exert cytotoxicity against tumor cells by IFN-γ and cytolytic granules. On the other hand, accumulating evidence suggested the IL12/IL12R could transduce the signal through the Fas_{TI} portion, thereby inducing apoptosis within the target cells.

To efficiently expression our fusion protein on tumor cells, lentivirus-based expression vectors were applied in this strategy. Promising viral titration of over $1x10^7$/ml was achieved as representative of efficient delivery approach. More importantly, to demonstrate the anti-tumor potential of IL12/Fas_{TI}, virally transduced Hela cervic carcinoma cells (Lent-IF/IL12/Fas) were cocultured with NK92 cell line and human

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PBMCs derived from donor blood, respectively. Results of both coculture models indicate increased IFN-γ production, enhanced NK cell activity, induced apoptosis as well as pronounced overall cytotoxicity in the Lent-IF treatment group. Therefore, it is worth noting that together with high-efficient gene delivery system such as lentiviral vectors, the novel fusion protein IL12/Fas_{TI} may act as a promising option for cancer treatments.

That being said, however, our study is still limited to many aspects. First, although our data confirmed that new-generation lentiviral system showed improved yield and efficiency, so far there have been few successful cases of lentivirus-based gene therapy in preclinical and/or clinical trials. One major challenge may come from the safety concerns. Even though high-yield viral titer can be accomplished by multiple methods, including ultracentrifugation and precipitation, it is nearly possible to make sure the purity of viral particles for clinical purpose. Moreover, it appears not potent enough by using one single fusion protein for cancer treatment. Fortunately, our lab has been working on multiple designs of chimeric proteins for cancer immunogene therapy. Therefore, an appropriate combination of fusions proteins delivered to the tumor site might contribute to a better outcome. Besides, to further explore the potential of novel fusion proteins, different tumor models in mice are required for the future work. Lastly, because tumor cells have the ability to gain resistance in almost any therapeutic setting, future study can be focused on if and how the tumor cells might escape the cytotoxicity.

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