12-2017

Interleukin-12/HFasTI: A Humanized Bifunctional Fusion Protein For Cancer Immunotherapy

Chelsea Anne Pennington-Krygier
Clemson University

Follow this and additional works at: https://tigerprints.clemson.edu/all_theses

Recommended Citation
Pennington-Krygier, Chelsea Anne, "Interleukin-12/HFasTI: A Humanized Bifunctional Fusion Protein For Cancer Immunotherapy" (2017). All Theses. 2777.
https://tigerprints.clemson.edu/all_theses/2777

This Thesis is brought to you for free and open access by the Theses at TigerPrints. It has been accepted for inclusion in All Theses by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.
INTERLEUKIN-12/HFASTI: A HUMANIZED BIFUNCTIONAL FUSION PROTEIN FOR CANCER IMMUNOTHERAPY

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

by
Chelsea Anne Pennington-Krygier
December 2017

Accepted by:
Dr. Yanzhang Wei, Committee Chair
Dr. Charlie Rice
Dr. Xianzhong Yu
ABSTRACT

Cancer remains a major health issue worldwide, and the leading cause of death in nearly half the states in America. As our understanding of the immune system has improved, so too has our ability to create and tailor immunotherapy and gene therapy for cancer treatment. Therapy with cytokines to boost immune activity against cancer cells has had limited success, but is largely restrained by the toxicity of systemic administration of the high doses needed to elicit the desired effect. Also limiting the effect of such cytokine therapy is the issue of cancerous cells already implementing tactics to evade immune detection, so increasing immune activity may still not wholly eliminate those cancerous cells. In the present study, a humanized fusion protein, IL-12/hFasTI, consisting of murine IL-12 (biologically active as an activator of human NK cells) and the transmembrane and intracellular domains of human Fas, was successfully created. The fusion gene was transfected into the human cell lines HEK293 and HeLa, and stable fusion-gene expressing clones were produced and verified by RT-PCR and immunohistochemistry. Preliminary data also show that the fusion protein is able to activate human NK cells and human peripheral blood mononuclear cells (PBMCs). When functionally confirmed in animal models, it may well provide a promising potential for a therapy applicable to many cancer types when coupled with a tumor-cell specific delivery system like nanoparticles or CRISPR/Cas9 technology.

Keywords: IL-12; NK cell; Fas; apoptosis; cancer
DEDICATION

I dedicate this work to my mother, Jerri Pennington, and my father, Dennis Krygier, who have always supported my pursuit of higher education and my goal to work in the field of cancer research.
ACKNOWLEDGMENTS

I would like to acknowledge and sincerely thank my advisor Dr. Yanzhang Wei for bringing me into his lab, and guiding and supporting me throughout my tenure as his graduate student. Thanks as well are due to Dr. Charlie Rice and Dr. Xianzhong Yu for serving on my committee. I would also like to thank my lab mate Iris Yang for her incredible help at the bench, whenever protocols needed fixing or my techniques needed improving.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>I. A REVIEW OF INTERLEUKIN-12 AND FAS ANTI-TUMOR IMMUNOTHERAPIES</td>
<td>1</td>
</tr>
<tr>
<td>Biology of cancer</td>
<td>1</td>
</tr>
<tr>
<td>Cancer therapies</td>
<td>4</td>
</tr>
<tr>
<td>Natural killer cells</td>
<td>9</td>
</tr>
<tr>
<td>IL-12 and its role in the immune system and cancer</td>
<td>11</td>
</tr>
<tr>
<td>Fas mediated apoptosis</td>
<td>13</td>
</tr>
<tr>
<td>Bi-functional fusion proteins in tumor therapy</td>
<td>15</td>
</tr>
<tr>
<td>IL-12 fusion proteins</td>
<td>16</td>
</tr>
<tr>
<td>II. AIMS OF THIS STUDY</td>
<td>18</td>
</tr>
<tr>
<td>III. INTERLEUKIN-12/HFASTI: A HUMANIZED BIFUNCTIONAL FUSION PROTEIN FOR CANCER IMMUNOTHERAPY</td>
<td>20</td>
</tr>
<tr>
<td>Abstract</td>
<td>20</td>
</tr>
<tr>
<td>Introduction</td>
<td>21</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>24</td>
</tr>
<tr>
<td>Results</td>
<td>29</td>
</tr>
<tr>
<td>Discussion</td>
<td>38</td>
</tr>
<tr>
<td>IV. CONCLUSION AND FUTURE WORKS</td>
<td>40</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>42</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Transfection and stable clone selection</td>
</tr>
</tbody>
</table>

## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Construction of fusion gene plasmid</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Construction of FullhFas control plasmid</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>RT-PCR verification of fusion gene transcription in stable selected clones</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
</tr>
</tbody>
</table>
|        | RT-PCR verification of FullhFas gene transcription in stable selected HeLa clones |...
| 5      | 34   |
|        | Verification of gene expression at protein level in HEK293 clones |...
| 6      | 35   |
|        | Verification of gene expression at protein level in HeLa clones |...
| 7      | 36   |
|        | Proliferation assay for selected HEK293 clones |...
| 8      | 37   |
|        | Live cells remaining post-coculture with NK or PBMCs |...|
CHAPTER ONE

A REVIEW OF INTERLEUKIN-12 AND FAS ANTI-TUMOR IMMUNOTHERAPIES

Biology of Cancer

A new diagnosis of cancer is expected in 1,688,780 Americans in the year 2017, with 600,920 patients dying of it during the course of year - these numbers do not include the estimated 63,410 females who will be diagnosed with breast cancer (breast carcinoma in situ) or the 74,680 patients who can expect a melanoma diagnosis. Of those 600,920 deaths, nearly half will be due just to cancers of the lung and bronchus, prostate/breast (depending on gender), and colorectum, and over a quarter of the total deaths will be due only to cancer of the lung. Though the cancer death rate has decreased twenty five percent since the early 1990’s, several cancers (such as uterine corpus and liver) have had increasing mortality rates, and cancer is now the most common cause of death in nearly half the states in America, as heart disease declines in those areas (Siegel et al. 2017).

Though cancer is a varied disease, differing not only between cancer types but even between different cells of the same tumor (Hanahan and Weinberg 2000), there are currently eight widely recognized hallmarks common to cancerous cells (Hanahan and Weinberg 2011). These hallmarks are sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, reprogramming energy metabolism, and evading immune destruction. As healthy cells undergo carcinogenesis, they will change
over the course of their generations, gaining these characteristics over time (Hanahan and Weinberg 2000).

These accumulating genetic changes may begin in common areas – mutations in tumor suppressor genes p53 (Hollstein et al. 1994) or Rb (Burkhart and Sage 2008) are both common across many types of cancers. However, a huge variety of different mutations can lead to transformation, and eventually, tumorigenesis, and different cells within a tumor will often have quite different genotypes as a result of these accumulating genetic changes occurring in different cells at different time points (Nguyen et al. 2012). In addition to these transformed cells, there is increasing evidence that some members of a tumor population will act as cancer stem cells (Nguyen et al. 2012). These cancer stem cells are hypothesized to be largely responsible for tumor metastasis as well as cancer therapy resistance, and are currently presumed to derive from normal stem cells (Lau et al. 2017). The characteristics of these CSCs means a successful eradication of cancer cells must also destroy these CSCs, in order to prevent cancer recurrence (Lau et al. 2017).

For over a century, it has been hypothesized that the immune system is able to detect cells undergoing the aforementioned neoplastic transformations (Ehrlich 1909). As the understanding of immunology and immune system surveillance developed, it became evident that the immune system can interfere in tumorigenesis and prevent the growth of tumors (Burnet 1970). However, as some tumor cells are destroyed, others are able to evade such a fate, causing the development of tumors with heightened immune evasion abilities later in the life of the host (Urban et al. 1982). This hypothesis became known as
cancer immunoediting, summarized into three phases with three E’s: elimination, equilibrium, and escape (Dunn et al. 2004). In the elimination phase, cancer cells are readily detected and killed by cells of the host immune system, both innate and adaptive (Girardi et al. 2001). The immune system is able to detect these early-phase cancer cells in various ways. One such method involves the cytokine interferon-γ (IFN-γ), secreted by a wide variety of immune cells, from macrophages and NK cells to helper and cytotoxic T cells. Endogenous IFN-γ binds to its receptor (expressed nearly ubiquitously on human cells) expressed on a cancer cell and causes signaling through the JAK-STAT pathway (Darnell et al. 1994). Ultimately, this increases the immunogenicity of that IFN-γ expressing cancer cell by activating innate immune cells (like NK cells) against the cancer cell and labeling it for destruction (Kaplan et al. 1998). NK cells can also identify and destroy early-phase cancer cells through other methods, including through the cancer cells’ lack of cell surface MHC expression, a lack which marks the cancer cell as aberrant enough to warrant lysis (Trinchieri 1989).

In the equilibrium phase, the population of cancer cells which have survived the elimination phase are in an equilibrium state with the immune system: not fully destroyed, but as a population, not completely resistant to killing, either. This phase is hypothesized to be the longest chronologically, as it is the phase where some cancer cells are being destroyed by the immune system, but others are able to escape destruction through various mutations (Dunn et al. 2002). By the end of this phase, the cancer cells which have survived have managed to do so by evading immune destruction via their decreased immunogenicity. Finally, in the escape phase, the cancer cells which have
survived destruction in the equilibrium phase will escape the detection and destruction at the hands of the immune system and begin to grow (Dunn et al. 2004).

As cancer cells proceed through this immunoediting process, evading and eventually escaping the killing of various immune cells, they become less and less immunogenic and therefore free to proliferate at will, causing the malignant disease we know as cancer (Dunn et al. 2004). Thus with the combination of the proliferative potential of cancer stem cells, and the decreased immunogenicity of the remaining cancer cell population, eliminating the entirety of that population becomes a tricky task.

**Cancer Therapies**

The primary burden of cancer therapies is to eliminate the population of cancer cells, while leaving healthy cells alone - or at least minimally damaged so that they are able to recover (Palumbo et al. 2013). As the focus of this paper lies in the creation of a bi-functional fusion protein for cancer therapy purposes, addressing the use of surgery as a cancer treatment will be omitted. Instead, we will begin the discussion of cancer therapies with chemotherapy.

*Chemotherapy* as a cancer treatment was originally hailed as a ‘magic bullet’ that would kill cancer cells and spare healthy ones – exactly what is sought from a cancer therapy. Perhaps the most appealing aspect of chemotherapy, wherein a patient is treated with a toxic chemical agent, was that these agents would have a greater effect on fast-growing, nutrient-greedy cancer cells (Palumbo et al. 2013). Chemotherapy drugs may have varying functions, including stabilizing microtubules (thereby halting mitosis in rapidly dividing cancer cells), acting as alkylating agents (blocking mitosis by alkylating
DNA), or antimetabolites (blocking the use of a metabolite). However, these modes of action will also wreak havoc on healthy but quick-growing tissues. As a result, systemic side effects ranging from nausea, vomiting, and fatigue, to neuropathy, stroke, and cardiac myopathy, are a serious risk (Palumbo et al. 2013). Even when successful, the use of these toxic agents can have detrimental long-term effects which may very well include the formation of a new cancer (Swerdlow et al. 2000).

**Radiation therapy** operates under much the same premise as chemotherapy: applying a cell-damaging element with the aim to neuter and eliminate quick-dividing cancer cells (Bernier et al. 2004). However, it does differ from chemotherapy in that radiation is applied to a tumor-localized region rather than the entire body, and recent advances in both tissue imaging (Wang et al. 2017) and metabolic activity assessment can further enhance radiation therapy by personalizing radiation dosage based on actively growing tumor areas (Ling et al. 2000). This level of tumor-mapped therapy means reducing not only systemic damage, as occurs with chemotherapy, but also minimizing damage to tumor-adjacent healthy tissues (Baumann et al. 2016). Radiation therapy is also commonly used in combination with chemotherapy and/or surgery as a better method of eliminating and controlling tumor growth (Baumann et al. 2016). As with chemotherapy, use of radiation as a cancer therapy carries an increased long-term risk of developing a secondary malignancy (Aisenberg et al. 1997)

**Immunotherapy** is a broad-branching term which may include a variety of treatments: cancer vaccines, antibody therapy, or cytokine therapy. These therapies,
though widely different in their design and implementation, share the common end goal
of utilizing a patient’s immune system to fight their own cancer (Couzin-Frankel 2013).

*Cancer vaccines*, much like typical prophylactic vaccines, utilize disease-specific
antigens to elicit an immune response. These vaccines rely on the identification of tumor
antigens, and their long-term success largely lies in the utilization of antigens which are
not only tumor-specific, but are critical to the malignancy of the tumor itself (Rosenberg
1999). A tumor-associated antigen which is phenotypic of the tumor but not necessarily
required for it to maintain malignancy means that through immunoediting, the tumor may
down-regulate the expression of that antigen and thus cause the vaccine to lose its
effectiveness (Rosenberg 1999). These cancer vaccines may contain patient- or cancer-
specific peptides, proteins, or DNA, and can elicit tumor regression with little to no side
effects; side effects can be more severe if the vaccine is combined with a cytokine
therapy like IL-2 (Rosenberg et al. 1998). Or, a cancer vaccine may be developed based
specifically on a patient’s specific tumor, and used to prime the patient’s own dendritic
cells or T cells, which can similarly result in minimal side effects (localized swelling or
redness, flu-like symptoms) with complete or partial tumor and metastases regressions
(Nestle et al. 1998).

*Antibody therapy* is a type of immunotherapy which utilizes monoclonal
antibodies (mAbs) to treat cancer instead of inherently toxic compounds. Originally these
mAbs were thought a failure due to the immunogenicity of murine mAbs themselves
when administered to the host as well as their quick clearing from the body, but once
humanized mAbs (chimerically designed murine mAbs with portions of human sequence)
were designed, these issues were eliminated (Weiner 2015). These humanized mAbs have many inherent benefits derived from their similarity to normally occurring human IgG antibodies – they have a long half-life as stable proteins, they are well tolerated by the immune system of a patient, and can interact favorably with that immune system as well (Weiner 2015). The target of these mAbs can be designed not only for a wide range of cancers and epitopes but also for different biological purposes (Weiner 2015; Rogers et al. 2014). The antigen to which the mAb binds would ideally be minimally expressed on healthy cells, overexpressed on cancer cells, and not likely to be present in soluble form in the body (Weiner 2015).

Once bound to a cancer cell, a therapeutic mAb can trigger complement-dependent cytotoxicity, whereby complement components are activated through the classical pathway to ultimately trigger cytotoxic killing and phagocytosis of the mAb-bound cell (Rogers et al. 2014). However, cancer cells may be protected from this method of killing due to their ‘self’-like surface expression of complement regulation proteins, and so antibody dependent cellular cytotoxicity (ADCC) may come into play instead (Rogers et al. 2014). ADCC, mediated largely by NK cells, results in an NK cell triggering its mAb-bound target to commit apoptosis as a result of the granules released by that NK cell, which also releases pro-inflammatory cytokines like IFN-γ (Rogers et al. 2014). The mAbs may also directly interfere with the signaling of cancer cells, sometimes resulting in signal-induced apoptosis (Pedersen et al. 2002). Side effects from mAb therapy are usually minimal and vary based on the antibody, but range from rash,
diarrhea, and hypertension, to perforation of the bowel or cardiac failure in a slim percentage of patients (Palumbo et al. 2013).

Cytokine therapy aims to stimulate an immune response against cancer cells, or may even aim for a more cell-specific response. For example, cytokine therapy can often aim to activate NK cells against the cancer cells they have learned to tolerate rather than destroy (Ardolino et al. 2014). In a murine tumor model wherein the cancer cells were MHC class I-deficient (meaning they should be recognized as abnormal and destroyed by a functional NK cell), the NK cells which infiltrated the MHC class I-deficient tumors actually became inactive. However, upon treatment with the NK-activating cytokines IL-12 and IL-18, or a modified IL-2, the NK cells regained their function and the mice displayed increased survival (Ardolino et al. 2014). For human metastatic renal cancer carcinoma, treatment with IL-2 and/or IFN-α can increase survival while decreasing tumor burden, and high-dose IL-2 treatment (approved by the FDA in 1992) has resulted in patients being cured of their renal cancer, with disease-free survival in some patients even 10 years after the conclusion of therapy (Atkins et al. 2004). There is also FDA approval for IFN-α as a treatment for melanoma and hairy cell leukemia, and IL-2 was approved for melanoma as well as its earlier uses against renal carcinoma (Weber et al. 2015). Though these therapies have great promise in that they activate the body’s own defenses against a cancer, they can be highly toxic as well: side effects ranging from flu-like symptoms, fatigue, and depression, to psychosis, liver toxicity, suicidal depression, and cardiac complications (Weber et al. 2015).
As our understanding of immunology and cancer itself have increased, so too has our ability to design cancer treatments which are cancer-cell specific and non-harmful to healthy cells. As always, however, there is room for improvement in the quest for highly specific and efficient cancer therapies.

**Natural Killer Cells**

As previously mentioned, natural killer cells are a vital part of the innate immune system and can directly kill their targets, like virus-infected or early-stage cancer cells (Waltzer et al. 2005). While cells of the adaptive immune system need antigen-specific binding to recognize and destroy their targets, NK cells recognize a much broader selection of ligands, expressed in common throughout the body rather than the unique epitope carefully selected for a B or T cell (Vivier et al. 2011). These NK-recognized ligands may be stimulatory or inhibitory, as the powerful cytolytic activity of the NK population must be kept carefully in check (Vivier et al. 2004). The response of an NK cell depends on which of those ligands binds to the various NK cell surface receptors. For example, MICA (MHC class I chain related protein A) binding to its NK cell expressed receptor, NKG2D, will signal to that NK cell that this MICA-expressing cell is under stress, as MICA is expressed only on intestinal cells, and is upregulated elsewhere as a stress response (Gasser and Raulet 2006). This MICA-NKG2D binding will activate the NK cell to kill the MICA-expressing cell as a response to its stress signal (Gasser and Raulet 2006). The NKG2D response is of particular interest for immunotherapy and cancer research, since cancer cells which are forced to express this ligand may prompt tumor eradication at the hands of NK cells. Mice were experimentally able to reject tumors
whose cells were designed to express NKG2D ligands, for example (Diefenbach et al. 2001).

NK cells kill their targets in one of several ways, when more activating signals than inhibiting are received through ligand binding between the target cell and NK cell. The activation of an NK cell will prompt it to form a cell-cell connection, called a synapse, with its target. Along this synapse, the NK cell will release granules containing perforin and granzyme proteases, which will enter the target cell and prompt it to commit apoptosis (Rogers et al. 2014). The NK cell will promote inflammation in the area by producing inflammatory cytokines like TNF-α and IFN-γ (Vivier et al. 2011). Alternatively, NK cells may induce apoptosis in target cells expressing the death-receptor Fas on their membranes, through Fas-ligand binding and activation of the apoptotic cascade which results from Fas receptor activation (Chua et al, 2004). Natural killer cells are also the primary effector of antibody-dependent cellular cytotoxicity (ADCC). ADCC of NK cells occurs when an NK cell encounters an antibody-opsonized cell, binds the Fc region of the IgG antibodies via the Fcγ receptor expressed on the NK cell, and then induces apoptosis and localized inflammation through the same method described earlier, by secretion of granules (containing perforin and granzymes) and inflammatory cytokines (Shevtsov and Multhoff, 2016).

NK cells can detect and destroy early-stage cancer cells within the body utilizing the methods here described, but they are also capable of eliminating later-stage solid tumors. This perforin-mediated killing can be accomplished upon NK cell infiltration into the tumor microenvironment (Liu et al. 2012). NK cell infiltration into a tumor can
therefore be a strong indicator for patient outcome, where high infiltration correlates to increased patient survival time or even tumor regression when combined with certain other therapies, such as a local treatment with NK-activating cytokine IL-12 (Baginska et al. 2013; van Herpen et al. 2005).

Despite the natural abilities to destroy target cells without antigen-specific priming for which NK cells were named, cancer cells can still evade NK cell killing, or even redirect it to protect the tumor. Cancer cells can shed NK-activating ligands or even transfer their NK-activating ligands onto an NK cell which forms a synapse with it via trogocytosis, resulting in the NK cell being targeted by other NK cells for destruction (Salih et al. 2002; Lopez-Soto et al. 2015). Shedding NK-activating ligands like MICA results in measurably elevated levels of those ligands in patient serum, and causes the corresponding activating receptor (here, NKG2D) to be endocytosed and degraded, even on T cells which also express that receptor (Groh et al. 2002). Even the hypoxic environment of a tumor itself can impact the ability of the NK cell population to effectively destroy cancer cells: hypoxic tumor microenvironments have been shown to impede the activation of NK cells by IFN-γ (Baginska et al. 2013). This suppression of NK cell activity indicates that an alteration of the tumor microenvironment, or an alteration of the NK cells themselves, may have promise in cancer therapy.

**IL12 and its Role in the Immune System and Cancer**

Interleukin 12 (IL-12) is a heterodimeric cytokine with a mass of 74 kDa, comprised of disulfide-bonded α- and β-chains, which are the p35 and p40 subunits, respectively (Colombo and Trinchieri 2002). Secreted by activated dendritic cells, B cells, and
macrophages, IL-12 promotes the activity of CD4 T cells, CD8 T cells, NK cells, as well as prompting the differentiation of Th1 cells (Klinke 2015; Vignali and Kuchroo 2012). The IL-12 cytokine can bind to IL12Rβ1 or IL12Rβ2, which are expressed on the surface of resting and activated NK cells and activated T cells (Robertson and Ritz 1996). Following IL-12 binding, the IL-12R induces tyrosine phosphorylation and activation of the JAK2 and TYK2 kinases, which then phosphorylate and activate STAT4. STAT4, activated only by IL-12 and IFN-α, is primarily responsible for the downstream functional activity of IL-12 in immunity (Colombo and Trinchieri 2002). This cytokine, as previously mentioned, has a powerful effect to activate NK cells and other white blood cells, especially in causing them to produce the pro-inflammatory cytokine IFN-γ (Robertson and Ritz 1996). IFN-γ secretion by these cells can also create a powerful pro-inflammation positive feedback loop, wherein IL-12 activation of Th1 cells causes IFN-γ secretion, which then prompts dendritic cells and macrophages to secrete IL-12 (Colombo and Trinchieri 2002).

In cancer models, mice given systemic doses of IL-12 experienced complete tumor regression, metastasis inhibition, and increased lifespan compared to untreated mice. They were later able to reject a follow-up specific tumor challenge, though this rejection was only seen if the same tumor was used; different tumor types were not rejected (Mu et al. 1995). In human clinical trials, as mentioned in the discussion of cancer therapies, systemic administration of immune-activating cytokines can come with dangerous toxic effects (Weber et al. 2015). With IL-12, these toxicities are often associated with only a marginal clinical effect and clinical trials have resulted in patient
deaths as a direct result of this treatment (Colombo and Trinchieri 2002). In murine studies this could be avoided by engineering either fibroblasts or tumor cells to locally secrete IL-12, as the tumor cells were prevented from growing due to immune activation against them, and the mice were able to reject new tumors when challenged (Robertson and Ritz 1996). This indicates that a therapy where cancer cells can be designed to express IL-12 within the tumor microenvironment does have promise as an immunotherapy, activating an immune response against the tumor without risking the systemic toxicity of direct IL-12 administration.

**Fas Mediated Apoptosis**

A member of the tumor necrosis factor receptor family, the membrane protein Fas, or CD95, is expressed throughout the body (Peter et al. 2015). The Fas receptor forms homotrimers on cell membranes, and the intracellular domains of each receptor contain a death domain (DD) necessary for the initiation of apoptosis (Siegel et al. 2000). The Fas ligand, CD95L (or CD178/TNFSF6) is expressed primarily on the surface of activated T and NK cells (Peter et al. 2015). When this ligand binds to the homotrimer receptor complex, the intracellular death domains of the Fas receptor form a DISC, or death-inducing signaling complex. The DDs form this DISC by recruiting an adapter molecule called FADD (Fas-associated death domain protein) which has its own DD, and then recruiting procaspase-8 into the DISC as well (Krammer 2000). With procaspase-8, other proteins, including procaspase-10 and the caspase regulator c-FLIP also associate with the DISC (Peter and Krammer 2003). Following DISC assembly, procaspase-8 is autoproteolytically cleaved and activated, releasing into the cytoplasm and cleaving other
proteins like procaspase-3 (Krammer 2000). The activation of this caspase cascade indicates the Fas-expressing cell is now committed to the apoptotic pathway, as the active caspases cleave crucial cellular components (Krammer 2000).

While Fas mediated apoptosis plays an essential role in healthy tissues and in the immune system in particular, cancer cells have a variety of methods to avoid this apoptotic pathway or even use it to their advantage within the tumor microenvironment (Igney and Krammer 2002). Cancer cells can evade Fas mediated apoptosis by secreting a soluble form of CD95 which lacks the typical transmembrane domain, thereby inhibiting normal CD95 signaling (Cheng et al. 1994). They may down-regulate expression of the Fas receptor, thereby protecting them from that apoptotic pathway, or bear a mutated Fas protein with a nonfunctional intracellular death domain or shortened extracellular receptor (Strand et al. 1996; Landowski et al. 1997). They may even express the FasL themselves, turning the Fas mediated apoptotic pathway against its effector cells and causing apoptosis in the cytotoxic cells rather than the cancer cells (Igney and Krammer 2005).

For those cancers which do express Fas, treatment with an anti-Fas antibody, meant to trigger Fas mediated apoptosis without the intervention of NK or T cells, causes often-lethal side effects (Peter et al. 2015; Ogasawara et al. 1993). These cytotoxic effects are particularly seen in the liver, where Fas expression is hypothesized to both play a role in liver regeneration and be the cause of death in the murine model (Ogasawara et al. 1993). However, in vitro experimentation where cancer cells are genetically altered to express a fusion protein – containing an extracellular domain other than Fas (such as
MULT1, the murine homolog of MICA, or IL-12), combined with the transmembrane and intracellular (death-domain containing) domain of Fas, shows promise as a potential cancer therapy option as the tumor cells not only commit apoptosis at an increased rate, but are also better killed by NK cells (Kotturi et al. 2008; Yang et al. 2016).

**Bi-Functional Fusion Proteins in Tumor Therapy**

In recent years, as molecular biology techniques have improved, the possibility to combine different proteins as a method of treating cancers has evolved. These fusion proteins have the benefit of combining the methods of action of both individual elements which comprise the fusion protein. A clinically hopeful fusion protein design involves fusing a cytokine, like IL-12 or IL-2, with a tumor-specific monoclonal antibody (Helguera et al. 2002). A fusion protein of this design has many benefits: it lasts longer in the body than the cytokine alone, reduces the risks typically seen with high-dose systemic cytokine treatments, and the antibody portion of the fusion protein is tumor-antigen specific, as well as acting as a therapeutic mAb in that it can trigger ADCC or NK cell mediated killing (Stamova et al. 2012). In addition to these benefits, the cytokine element of the fusion protein is still able to increase the immune response to the cancer, but does so within the localized tumor microenvironment, while also hopefully increasing the immunogenicity of the cancer cells via the antibody portion of the protein (Helguera et al. 2002).

Several of these fusion proteins have been designed. One study found that when the Fab fragment (the antigen-binding fragment of an antibody) of a tumor-specific antibody protein was conjugated to a NK-activating ligand like MICA (discussed
previously), the fusion proteins were able to efficiently coat their respective tumor cells and, following coating, the tumor cells were subject to increased levels of NK-cell mediated lysis (Germain et al. 2005). These fusion proteins were successful in vitro for human cancer cell lines when utilizing Fabs of anti-CD20 targeting non-Hodgkin lymphoma or anti-HER2 targeting breast cancer (Germain et al. 2005). However, these fusion proteins are not limited only to expressing NK-specific ligands – several fusion proteins have been derived which contain cytokines instead.

**IL-12 Fusion Proteins**

Particularly relevant to this discussion are IL-12 fusion proteins. These take advantage of interleukin-12’s function as an activator of cytotoxic T cells and NK cells (Klinke 2015; Vignali and Kuchroo 2012). The majority of these fusion proteins would utilize the same tumor specificity of the aforementioned fusion proteins, as they are composed of some tumor antigen specific antibody region fusion to a fragment of IL-12 or a recombinant form of IL-12 (Penichet and Morrison 2001). Because the IL-12 portions of these proteins activate T cells, they are potentially able to create a measurable tumor-specific immune memory as a result of that T cell activation, in addition to increased tumor cell death as a result of NK cell activation (Penichet and Morrison 2001).

Another method of utilizing IL-12 as a fusion protein element involves utilizing IL-12’s extracellular binding to immune cells to send a second, different signal to a tumor cell by creating a fusion protein which is transfected into the tumor cell itself rather than administered therapeutically. A fusion protein combining IL-12 and MICA (an NK activating ligand for the NKG2D receptor), when transfected into human tumor cells, was
able to activate NK cells and increase their IFN-γ production (Tietje et al. 2017). A murine fusion protein, containing IL-12 as a membrane anchored (rather than soluble) protein combined with the transmembrane and intracellular domains of the Fas receptor, once transfected into murine cancer cell line TC-1, was able to not only activate NK cells via the IL-12 portion of the protein, but was also able to prompt cancer cells to commit apoptosis as a result of the transmembrane and intracellular Fas domains (Yang et al. 2016). It is clear that fusion proteins containing some element of IL-12 are effective at activating NK cells against tumor cells.

Based on the review above, it is clear that (i) cancer is a leading cause of mortality in developed nations with a capacity to mutate and avoid immune detection; (ii) NK cells play a crucial role in protecting hosts from tumor development through induction of apoptosis; (iii) the cytokine IL-12 activates NK cells to better detect and destroy tumor cells; (iv) Fas is an important death receptor and the Fas/FasL pathway induces apoptosis in Fas-expressing cells; and (v) multi-functional fusion proteins can effectively combat cancer by combining the functionality of various proteins without the risks of systemic administration of the single components alone.
CHAPTER TWO
AIMS OF THIS STUDY

As the first step of this project, a fusion gene encoding mouse IL-12 (sequentially similar to human IL-12 and efficacious in activating human NK cells) and human FasTI will be constructed in the pcDNA3.1zeo(+) vector, and transfected into two human cell lines, embryonic kidney cell line HEK293, and cervical carcinoma cell line HeLa. After stable clones are established, the expression of the fusion gene will be investigated in vitro with RT-PCR and immunohistochemistry. The function of the fusion protein will be evaluated by enhanced NK/PBMC cytotoxicity of tumor cells in a co-culture setting.

1. Construction of the fusion gene IL-12/hFasTI

For the fusion gene, the extracellular domain of mouse IL-12, and the transmembrane and intracellular (TI) domains of human Fas (hereafter called ‘hFasTI’), will be ligated into the expression vector pcDNA3.1/zeo(+), with a GGGS3 linker between the two portions of the fusion gene. Control vectors will be constructed which contain a membrane-anchored mouse IL-12 sequence, or the full human Fas (hereafter called ‘FullhFas’) sequence. The mIL-12 will be utilized instead of the human IL-12 as it is readily available due to prior projects in the lab, and is sequentially similar to human IL-12 and able to stimulate human NK cells.

2. Transfection and selection of stable clones

To test the efficacy of the fusion gene and measure its tumor-killing and immune-activating effects, the fusion and control gene expression vectors will be transfected into human embryonic kidney (HEK293) and cervical cancer (HeLa) cells using
Lipofectamine 2000. Transfected clones will be selected using zeocin resistance as a selection marker.

3. Characterization of stable clones by RT-PCR and immunohistochemistry

Stable clones will have total RNA extracted, from which a cDNA library will be compiled. These cDNA libraries will be used to run RT-PCR to verify the transcription of a control or fusion gene. To verify the successful translation of the control or fusion gene, antibody staining will be utilized to detect the extracellular domains of the control or fusion gene.

4. Fusion gene function

To confirm that the fusion protein is effective as an anti-tumor protein, an in vitro functional assay will be conducted to measure cytotoxicity of NK cells or peripheral blood mononuclear cells (PBMCs) on stable clones using an MTS proliferation assay.
CHAPTER THREE

INTERLEUKIN-12/HFASTI: A HUMANIZED BIFUNCTIONAL FUSION PROTEIN FOR CANCER IMMUNOTHERAPY

Abstract

Cancer treatments with cytokines is meant to boost the immune system’s recognition of cancer cells, but carries risks of systemic toxicity with high doses of cytokine administration. These treatments can be promising in that they can prompt cytotoxic cells of the immune system, like natural killer cells, to once again recognize and destroy cancer cells. However, late-stage cancer cells not only evade recognition by lymphocytes like NK cells, but they can also evade Fas-mediated apoptosis by modifying, or completely abolishing their expression of the Fas receptor. In this study, we designed and evaluated the functionality of a human fusion protein, IL-12/hFasTI, which consists of membrane anchored IL-12 and the transmembrane and intracellular domains of human Fas. Previous studies with the murine version of this protein proved highly promising. The humanized fusion construct was transfected into human embryonic kidney cell line HEK293 and cervical carcinoma cell line HeLa. Stable cell clones expressing the fusion protein were grown and verified using RT-PCR and immunohistochemistry. Preliminary data also show that the fusion protein is able to activate human NK cells and PBMCs. When functionally confirmed in animal models, this may well provide a promising potential for a therapy applicable to many cancer types when coupled with a tumor-cell specific delivery system like nanoparticles or CRISPR/Cas9 technology.

*Keywords*: cancer; IL-12; NK cell; Fas; apoptosis
Introduction

A crucial part of the innate system is natural killer (NK) cells, which identify and kill virus-infected or early-stage cancer cells (Waltzer T et al. 2005). Though adaptive immune cells need antigen-specific binding to recognize and destroy their targets, NK cells recognize a much broader selection of ligands, rather than the unique epitope recognized by a B or T cells (Vivier E et al. 2011). These recognized ligands may be stimulatory or inhibitory, as the powerful cytolytic activity of the NK cell population must be kept carefully regulated to respond to threats to host health (Viver E et al. 2004).

Activating ligands, rather than being recognized for a specific epitope, are rather recognized like distress signals, upregulated upon cellular stress, like MHC class I chain related protein A, or MICA (Robertson M and Ritz J 1996; Gasser S and Raulet DH 2006). Following NK cell recognition of a target cell, NK cells induce apoptosis of the infected or cancerous cell via , while also secreting inflammatory cytokines like interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) to promote further immune activity in the area (Waltzer T et al. 2005).

Interleukin-12 (IL-12) is an inflammatory cytokine that acts as a powerful stimulator of NK cells and other cytotoxic lymphocytes (Klinke DJ 2015). The IL-12 receptor is expressed on NK cells and activated T cells (Robertson MJ and Ritz J 1996). Upon IL-12 binding, the IL-12R will activate the JAK2/STAT4 pathway, which induces downstream immune activity like IFN-γ secretion (Colombo MP and Trinchieri G 2002; Robertson MJ and Ritz J 1996). IFN-γ is another powerful pro-inflammatory cytokine, and the secretion of IFN-γ by IL-12 activated cells creates a powerful pro-inflammation
positive feedback loop, wherein a subset of IL-12 activated T cells secrete IFN-γ, prompting other leukocytes like dendritic cells and macrophages to secrete IL-12 (Robertson MJ and Ritz J 1996; Colombo MP and Trinchieri G 2002).

Fas, or CD95, is a cell surface death receptor expressed throughout the body which triggers apoptosis in the receptor-expressing cell upon binding with its ligand (Peter ME et al. 2015; Romagne F and Vivier E 2011). The CD95/Fas ligand (CD95L/FasL) is primarily expressed on the surface of activated T and NK cells, which can induce target cells to commit apoptosis when biologically necessary by activating a cytoplasmic death domain and downstream caspase cascade (Peter ME et al. 2015; Krammer PH 2000). Though this Fas mediated apoptosis plays an essential role in healthy tissues and in the immune system in particular, cancer cells are often able to avoid this pathway or even use it to their advantage (Igney FH and Krammer PH 2002). They can secrete a soluble form of CD95 to inhibit normal CD95 signaling within the tumor microenvironment, or express a mutated Fas receptor that bears a non-functional death domain or shortened extracellular domain (Strand S et al. 1996; Landowski TH et al. 1997).

Utilizing Fas or IL-12 as cancer therapies is daunting, as systemic treatment with an anti-Fas antibody can be lethal and systemic doses of IL-12 high enough to result in clinically significant tumor reduction can be toxic as well (Weber JS et al. 2015; Ogasawara J et al. 1993). However, combining these two elements into a fusion protein can reduce or eliminate the risks of either element alone, while also combining the benefits of each individual protein. When murine tumor cells were transfected with a Fas
fusion protein, wherein a murine NK-activating ligand (MULT1) was fused to the transmembrane and intracellular domains of the murine Fas receptor, the fusion protein was able to successfully deliver intracellular signaling and activation of the Fas-mediated apoptotic pathway after NK cell binding to the extracellular MULT1 ligand (Kotturi H et al. 2008). Another murine fusion protein, comprised of membrane-anchored IL-12 and the same transmembrane and intracellular domains of Fas (IL-12/FasTI), when transfected into murine cancer cell line TC-1, was able to both activate NK cells via the IL-12 domain and also prompt the fusion protein expressing cells to commit apoptosis via the FasTI domains of the protein as well (Yang X et al. 2016).

Based on the success of the latter murine fusion protein, here we propose constructing and testing the efficacy of a humanized version. This fusion gene, IL-12/hFasTI, will combine membrane anchored IL-12 with a human version of the transmembrane and intracellular domains of Fas. We hypothesize that human cells expressing the fusion protein will not only activate NK cells against cancer cells via the IL-12/IL-12R pathway, but also induce apoptosis of the cancer cells via the Fas mediated apoptotic pathway. These fusion genes contribute to the field of cancer immunotherapy and gene therapy, as the membrane bound IL-12 portion of the gene will be able to remain at high levels within the tumor microenvironment, without the systemic toxicity of a similar concentration of soluble cytokine. Furthermore, because the fusion protein act primarily upon the innate immune system, they are not specific to particular tumor antigens, and should be applicable in many different cancer types.
**Materials and Methods**

**Construction of pcDNA3.1 Zeo (+)/IL-12/hFasTI vector**

A frozen sample of existing pcDNA3.1 Zeo (+) IL-12/mFasTI will be transformed into chemically competent *E. coli* and then collected using a plasmid miniprep kit (Qiagen, Valencia, CA, USA). The continued presence of the desired plasmid vector will be verified by a double restriction enzyme digest with the HindIII and EcoRI enzymes, which will cut the vector in two places. The digested vector will be run on an agarose gel for gel electrophoresis, and when visualized with UV light will show DNA fragments of approximately 5000 and 2000 base pairs - representing the pcDNA3.1zeo(+) vector and the IL-12/mFasTI fragment of interest, respectively. Following confirmation of the complete expression vector’s presence, PCR will be run using the pcDNA3.1zeo(+)MICA/hFasTI vector as a template, to amplify a new human FasTI sequence which does not include the (GGGS)3 linker sequence, as the existing IL-12/mFasTI vector already contains the (GGGS)3 linker at the 3’ end of the IL-12 sequence, upstream of the BamHI cut site. The 5’ forward primer (CGGGATCCAGATCTAACTTGGGG) will begin with a BamHI restriction enzyme cut site (GGATCC), followed by the beginning of the FasTI sequence. The 3’ reverse primer (CGGAATTCCTGGACCAAGCTTTGGATTTCATTTC) will contain the reverse complementary sequence for the EcoRI restriction enzyme cut site (GAATTC), which is at the 3’ end of the hFasTI fragment, and the 3’ sequence of the FasTI fragment. PCR will be performed using the Phusion High-Fidelity PCR kit (New England BioLabs, Ipswich, MA, USA). The PCR fragment will be extracted and purified using an agarose
gel and purification kit (Qiagen, Valencia, CA, USA). Following purification, the Fas fragment and the pcDNA3.1zeo(+)/IL-12/mFas vector will be digested by a double enzyme digest with EcoRI and BamHI, and extracted from the agarose gel and purified using the Qiagen gel purification kit. The digested vector and Fas fragment will be ligated together using the Fast-Link DNA Ligation kit (Epicentre Biotechnologies, Madison, WI, USA) to create the plasmid pcDNA3.1zeo(+)/IL-12/linker/hFasTI. To confirm that the sequence is as predicted, the new fusion plasmid will be sent to CUGI for sequencing.

**Construction of pcDNA3.1zeo(+)/FullhFas vector**

The IL-12 pDisplay control vector is already assembled in the pcDNA3.1zeo(+) vector, from previous work completed in this lab. As such, no further cloning is required in the construction of the IL-12 control vector.

The Fas control vector will contain the complete (extracellular, transmembrane, and intracellular domains) Fas sequence (referred to as FullhFas here, as opposed to the fusion gene which contains only hFasTI, the transmembrane and intracellular domains). As such, the FullhFas sequence will be amplified from pMD18-T/human CD95 which contains the full Fas sequence, using 5’ primer GGGCTAGCATGCTGGGCATCTGGACCCT with a NheI restriction cutting site and 3’ primer GGCGGAATTCCTAGACCAAGCTTTGGATTT with an EcoRI restriction cutting site by PCR. The fragment will be excised and gel purified using the gel purification kit (Qiagen, Valencia, CA, USA). Double enzyme digestion will be
performed on both the FullhFas fragment and the pcDNA3.1zeo(+)/mIL12/hFasTI fusion plasmid, with NheI and EcoRI, allowing the insertion of the FullhFas sequence into the pcDNA3.1zeo(+) vector. The enzyme digested FullhFas sequence and pcDNA3.1zeo(+) vector will be ligated to create the plasmid pcDNA3.1zeo(+)/FullhFas. This ligation was confirmed by double restriction enzyme digest and DNA sequencing at Clemson University Genomics Institute.

Cells
Human embryonic kidney cells HEK293 (ATCC No. CRL-1573) were cultured in RPMI1640 media with 10% fetal bovine serum and 100ug/ml gentamicin at 37°C with 5% CO2. Human cervical cancer cell line HeLa (ATCC No. CCL-2) were cultured in the same growth media and incubation conditions.

Transfection
HEK293 and HeLa cells were transfected with either pcDNA3.1zeo(+)IL-12/linker/hFasTI, pcDNA3.1zeo(+)/FullhFas, or pcDNA3.1zeo(+)/IL-12, using Lipofectamine 2000 (Invitrogen) according to manufacturer instructions. To select for successfully transfected clones, the cells were subsequently exposed to a selecting media containing 100ug/ml zeocin. Cells which survived exposure to zeocin were allowed to grow out until they achieved a visible colony, at which point several colonies were selected from each transformation and sub-cultured in the selecting media.
RT-PCR

Total RNA was extracted from each HEK293 and HeLa IL-12/hFasTI, IL-12, and FullhFas clone using an RNeasy Plus kit from Qiagen following the manufacturer’s directions. cDNAs were produced from the total RNA using the Phusion RT-PCR kit from Thermo Scientific following the manufacturer’s directions (Thermo Scientific, Waltham, MA, USA). Equalized quantities of the DNA product was then added to a PCR reaction containing primers amplifying a 1064bp sequence (for the IL-12/hFasTI and IL-12 clones) or 1008 bp of hFas (for the FullhFas clones). A Phusion High-Fidelity DNA polymerase kit was used with the 5’ primer GCAGTGACATGTGGAATGGC and 3’ primer GGCGGAATTACCAGACCAAGCTTTGGGATT for the IL-12/hFasTI and IL-12 clones and 5’ primer GGGCTAGCATGCTGGGCATCTGGACCCCT and 3’ primer GGCGGAATTACCATGACCAAGCTTTGGGATT for the FullhFas clones (New England BioLabs, Ipswich, MA, USA). The beta-actin housekeeping gene was also amplified as a loading control for each HEK293 and HeLa clone.

Cell surface staining

Untransfected HEK293 and HeLa cells and a representative clone of HEK293/IL-12/hFasTI, HEK293/IL-12, HeLa/IL-12/hFasTI, and HeLa/IL-12 were stained using rat anti-mouse IL-12 antibody according to manufacturer instructions using the HRP-AEC system (R & D Systems, Minneapolis, MN, USA). The stained cells were analyzed using a fluorescent microscopic system (Olympus 1x70 fluorescent microscope).
Proliferation assay

To determine if the transfected clones grew at the same rate as untransfected cells, cell proliferation was checked with the Promega CellTiter 96\textsubscript{Nleous} nonRadioactive Cell Proliferation (MTS) assay every 24 hours for 5 days. On day one, 0.04x10\textsuperscript{6} cells of untransfected parental cells and a representative clone of each transfection were plated on 12-well-plates, into 15 wells. At each time point, 3 wells of each clone were tested with Promega’s CellTiter 96\textsubscript{Nleous} nonRadioactive Cell Proliferation (MTS) Assay. Clones were compared using a one-way ANOVA with Tukey’s post-test.

Cytotoxicity

Untransfected HEK293 cells and representative clones of HEK293/IL-12/hFasTI and HEK293/IL-12, were plated in a 96 well plate in triplicate at 0.2 x 10\textsuperscript{5} cells per well. 24 hours after plating, culture media was replaced with PBMC media and PBMCs were added to coculture wells at 1 x 10\textsuperscript{5} cells per well. Cells were cocultured for 48 hours, following which the 96 well plate was centrifuged to anchor the PBMCs, supernatant was removed, and then PBMCs were removed also via gentle washing. The amount of live clone cells remaining was determined using Promega’s CellTiter 96\textsubscript{Nleous} nonRadioactive cell proliferation assay following the manufacturer’s instructions. Clones were compared using a one-way ANOVA with Tukey’s post-test. Identical coculture was also run with NK92 cells, with coculture time reduced to 2 hours.
Results

Fusion gene and control plasmid constructions

(a) Plasmid pcDNA3.1zeo+/mIL12/hFasTI construction

The transmembrane and intracellular domain of the human Fas sequence was amplified from a fusion plasmid constructed earlier in our lab: pcDNA3.1zeo+/MICA/hFasTI using PCR with 5’ primer (CGGGATCCAGATCTAACTTGGGG) containing a BamHI cutting site and 3’ primer (CGGAATTCCTGGACCAAGCTTTGGATTTCATTTC) containing an EcoRI cutting site. The PCR fragment was then gel purified and extracted using a gel purification kit. In order to obtain the extracellular mIL12 sequence and GGGS3 linker sequence which would be upstream of the hFasTI fragment, a plasmid previously constructed in our lab was used: pcDNA3.1zeo+/mIL12/GGGS3/mFasTI. This plasmid was digested with BamHI and EcoRI, which excised the mFasTI sequence from the plasmid. After gel purification and extraction, the pcDNA3.1zeo+/mIL12/GGGS3 vector was ligated with the hFasTI fragment, to create the plasmid pcDNA3.1zeo+/mIL12/GGGS3/hFasTI. The success of the ligation was confirmed by double restriction enzyme digest and DNA sequencing at Clemson University Genomics Institute (Figure 1).

(b) Plasmid pcDNA3.1zeo+/FullhFas construction

Full length human hFas (FullhFas) cDNA was cloned from pMD18-T/human CD95, using PCR with 5’ primer (GGGCTAGCATGCTGGGCATCTGGACCCT) containing an Nhel cutting site and 3’ primer (GGCGGAATTCCTAGACCAAGCTTTGGATTTC)
Figure 1: Construction of fusion gene plasmid. Construction of fusion plasmid pcDNA3.1zeo(+)/IL12/hFasTI, by ligating existing pcDNA3.1zeo(+)/IL12 vector with PCR amplified hFasTI fragment.

containing an EcoRI cutting site. The PCR fragment was then gel purified and extracted using a gel purification kit. The pcDNA3.1zeo+ vector backbone was purified from the fusion plasmid constructed earlier in this study: pcDNA3.1zeo+/mIL12/hFasTI, which was double enzyme digested with NheI and EcoRI. After the PCR fragment of FullhFas was similarly digested, it was ligated to the NheI/EcoRI digested pcDNA3.1 vector to create the plasmid pcDNA3.1zeo+/FullhFas. This ligation was confirmed by double restriction enzyme digest and DNA sequencing at Clemson University Genomics Institute (Figure 2).
Stable clone establishment

HEK293 human embryonic kidney cells and HeLa human cervical carcinoma cells were transfected using Lipofectamine2000, with pcDNA3.1zeo+/mIL12 or pcDNA3.1zeo+/mIL12/GGGS3/hFasTI. HeLa cells were additionally transfected with pcDNA3.1zeo+/FullhFas. Zeocin resistant clones for each transfection were selected and sub-cultured (Table 1). HEK293 transfection produced six stable fusion clones, and four stable IL12 clones. HeLa transfection produced three stable fusion clones, two stable IL12 clones, and three stable FullhFas clones.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Zeocin Selection Level</th>
<th>Transfection Attempt</th>
<th>DNA: Lipofectamine2000 Ratio</th>
<th>Colonies Picked</th>
<th>Stable Clones Grown</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>200ug/ml</td>
<td>1 (4.30.2016)</td>
<td>3ug:10ul</td>
<td>9 fusion</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (6.15.2016)</td>
<td>2ug:4ul in 12-well</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400ug/ml</td>
<td>3 (7.27.2016)</td>
<td>4ug:10ul</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 (9.6.2016)</td>
<td>3ug:10ul</td>
<td>5 fusion, 7 IL12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 (10.15.2016)</td>
<td>2.5ug:5ul Lipofectamine3000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 (11.6.2016)</td>
<td>2.5ug:10ul</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DU145</td>
<td>100ug/ml</td>
<td>1 (3.21.2017)</td>
<td>2.5ug:10ul</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (4.13.2017)</td>
<td>2.5ug:5ul</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HEK293</td>
<td>100ug/ml</td>
<td>1 (11.6.2016)</td>
<td>2.5ug:10ul</td>
<td>16 fusion, 16 IL12</td>
<td>6 fusion, 4 IL12</td>
</tr>
<tr>
<td>HeLa</td>
<td>100-200ug/ml</td>
<td>1 (5.20.2017)</td>
<td>5ug:7.5ul</td>
<td>29 IL12, 32 FullhFas</td>
<td>2 IL12, 3 FullhFas</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (6.15.2017), fusion only</td>
<td>5ug:7.5ul</td>
<td>36 fusion</td>
<td>3 fusion</td>
</tr>
</tbody>
</table>

Table 1: Transfection and stable clone selection. All transfection attempts occurred in 6-well plates with Lipofectamine2000 reagent unless stated otherwise.

Characterization of stable clones by RT-PCR and immunohistochemistry

Two HEK293 fusion clones and one IL12 clone were selected for RT-PCR, which indicated that both fusion clones (IL12/hFasTI-3 and -9) were positive for the fusion gene while the IL12 control clone (IL12-5) and parental HEK293 cells were not (Figure 3a).

All three HeLa fusion clones were selected for RT-PCR, and clones 2 and 3 were positive for the fusion gene, while clone 1 and the parental HeLa cells were not (Figure 3b).

Similarly, all three FullhFas HeLa clones were selected for FullhFas gene expression
verification by RT-PCR. Clones 3 and 5 were positive for FullhFas expression, while clone 1 and the parental HeLa cells were not (Figure 4).

**Figure 3: RT-PCR verification of fusion gene transcription in stable selected clones.** For each PCR reaction, pcDNA3.1zeo/IL-12/hFasTI is denoted with “PL”. Housekeeping gene β-actin used as loading control. (a) Transcription of fusion gene in selected stable HEK293 clones (IL12-5, IL12-hFasTI-3, IL12-hFasTI-9), and parental HEK293 cells. Primers to amplify a 1064bp sequence containing IL-12 upstream of the GGGS3 linker, the linker itself, and the entire hFasTI portions of the fusion protein. (b) Transcription of fusion gene in selected stable HeLa clones (IL12-hFasTI-1, -2, -3). Same primers and PCR conditions used as part (a).

**Figure 4: RT-PCR verification of FullhFas gene transcription in stable selected HeLa clones.** For each PCR reaction, pcDNA3.1zeo/FullhFas is denoted with “PL”. Housekeeping gene β-actin used as loading control. Transcription of Fas gene in FullhFas control clones, using FullhFas specific primers in the clone lanes to amplify the entire 1008bp of the FullhFas sequence.

The same three selected HEK293 clones were also used for protein expression study using immunohistochemistry (IHC). IHC results demonstrated that IL12-5 and
IL12/hFasTI-3 expressed high levels of IL12 on their cell surfaces compared to control HEK293 cells, while IL12/hFasTI-9 expressed only very low levels of IL12 (Figure 5).

<table>
<thead>
<tr>
<th></th>
<th>Isotype Control</th>
<th>Anti-mouse IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>HEK293 IL-12 clone 5</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>HEK293 IL-12/hFasTI clone 3</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>HEK293 IL-12/hFasTI clone 9</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 5: Verification of gene expression at protein level in HEK293 clones. Cells from HEK293 clones IL12-5, IL12/hFasTI-3, IL12/hFasTI-9, and parental HEK293, were collected and cytospun onto glass slides. Cells were fixed and stained with anti-mouse IL12 antibody or isotype control antibody followed by anti-mouse IgG secondary antibody conjugated with HRP. The cells were then treated with DAB and observed under the microscope.

HeLa clones IL12-1 and IL12/hFasTI-2 were selected for protein expression study using IHC, and results demonstrated that IL12-1 and IL12/hFasTI-2 both expressed high levels of IL12 on their cell surfaces compared to control HeLa cells (Figure 6).
Figure 6: Verification of gene expression at protein level in HeLa clones. Cells from HeLa clones IL12-1, IL12/hFasTI-2, and parental HeLa, were collected and cyospun onto glass slides. Cells were fixated and stained with anti-mouse IL12 antibody or isotype control antibody followed by anti-mouse IgG secondary antibody conjugated with HRP. The cells were then treated with DAB and observed under the microscope.

Proliferation assay for selected clones

Selected HEK293 clones IL12-5 (IL12-5) and IL12/hFasTI-3 (IF-3) and parental HEK293 cells were plated in triplicate in 15-well plates to compare proliferation rates of transfectants versus parental cells. At each time point, cell proliferation was measured using an MTS assay for each clone in triplicate. Upon clone comparison using one-way ANOVA with Tukey’s post-test, there was no statistical significance in the proliferation rates of the transfected cells or parental cells (Figure 7).
Figure 7: Proliferation assay for selected HEK293 clones. 0.4x10^6 cells of each clone were plated in triplicate on four 15-well plates on day 1, and proliferation of clones was measured using MTS assay on one 15-well plate at each time point.

Tumor cell cytotoxicity

To confirm the hypothesis that ligand binding to the IL12 portion of the fusion protein can induce death signals into transfected cells through the hFasTI fragment of the fusion protein, HEK293 cells and clones IL12-5 and IL12/hFasTI-3 were cocultured for 2 hours with NK92 cells and an MTS assay was used to determine the relative quantities of live cells remaining, and a one-way ANOVA with Tukey’s post-test was used to analyze for statistical significance. Although not statistically significant, after coculture with NK cells, both clones of IL-12 and IL-12/hFasTI showed less living cells compared to HEK293 cells (Figure 8a). An identical coculture was run using human PBMCs instead of NK92 cells, with the coculture time increased to 48 hours. Again, both clones of IL-12 and IL-12/hFasTI showed a trend of less living cells compared to HEK293 cells (Figure 8b). However, after PBMC coculture there was a statistically significant increase in cell numbers for the parental HEK293 cells (p<0.05).
Figure 8: Live cells remaining post-coculture with NKs or PBMCs, where + indicates a coculture and cell line alone indicates identical culture conditions without the addition of NKs or PBMCs. (a) HEK293 clones were cocultured for 2 hours with NK92 cells, which were removed before clone proliferation was measured with MTS assay. One-way ANOVA with Tukey’s post-test showed no statistical significance between treatments. (b) HEK293 clones were cocultured for 48 hours with PBMCs, which were removed before proliferation was measured with MTS assay. One-way ANOVA with Tukey’s post-test showed no statistical significance between treatments, except for the parental HEK293 coculture, which had a significant increase of live cells compared to HEK293 cells alone (p<0.05).
Discussion

We have utilized molecular cloning and human cell lines to create and transfect a fusion-gene expressing plasmid, which codes for a bifunctional fusion protein with membrane-anchored IL-12 fused to the transmembrane and intracellular (TI) domains of the human Fas gene (Fig. 1). Following transfection into the human cell lines HEK293 and HeLa, expression of the fusion gene was confirmed at the RNA level via RT-PCR (Fig. 3, 4) and the protein level via immunohistochemistry (Fig. 5, 6). Proliferation rates of HEK293 clones were found to be similar enough to parental cells (Fig. 7) that coculturing with killer cells was able to proceed. Preliminary functional results indicated that though coculture with NK92 cells or PBMCs resulted in no statistically significant change in clone numbers post-cocultures (Fig. 8), there was a slight trend of decreased numbers of fusion clones and IL-12 clones post-coculture. PBMC coculture also resulted in a significant increase in parental HEK293 cells post-coculture (Fig. 8b). This increase could be the result of inadequate PBMC removal post-coculture, as rinsing to remove PBMCs had to be done very gently to avoid detaching the loosely-adherent HEK293 cells. All HEK293 cells, both parental and transfected clones, were easily detached from their culture surface, and this ease of detachment coupled with overly-gentle rinsing could obscure results for live cells post-coculture.

The humanized IL-12/hFasTI fusion protein would make a useful immuno/gene therapy agent in a variety of cancers, as it theoretically activates two pathways of cancer cell death which are naturally avoided by advanced tumors. The IL-12 region of the fusion protein activating NK cells within the tumor microenvironment would help those
NK cells regain their cancer-killing abilities, despite tumors often evading or shutting down this activity (Lopez-Soto A et al. 2014; Groh V et al. 2002). The activation of these NK cells within the localized tumor microenvironment would also mean that a therapeutic fusion-gene transfection would not need to be 100% effective, as the presence of the IL-12 on some tumor cells should be sufficient to activate NK cells against the tumor as a whole.

The Fas portion of the fusion protein would also allow reactivation of a cancer elimination method which prevents most transformed cells from becoming full-blown cancer: Fas mediated apoptosis. By combining the fully functional TI domains of the Fas protein with a non-Fas extracellular signal, this fusion protein can reactivate the Fas apoptosis pathway which many cancers evade via truncation or mutation of the Fas protein (Strand S et al. 1996; Landowski TH et al. 1997). Instead of requiring activated T or NK cells to induce Fas/FasL signaling, this fusion protein can induce the apoptotic cascade upon binding to IL-12 (Peter ME et al. 2015). This also circumvents the immune evasion method of tumors whereby they secrete soluble Fas (Cheng J et al. 1994).

This bi-functional human fusion protein thereby will induce apoptosis of treated cells, and activation of natural killer to further reduce a tumor cell population, through its human FasTI and IL-12 domains, respectively.
CHAPTER FOUR

CONCLUSION AND FUTURE WORKS

The fusion gene IL-12/hFasTI, containing the sequence for an extracellular membrane-anchored Interleukin-12 domain, and the transmembrane and intracellular (TI) domains of the human Fas death receptor, was constructed within the pcDNA3.1/zeo(+) expression vector. The IL-12 domain acts as an activator for NK cells, and the FasTI domains retain their apoptosis-inducing activity upon extracellular signaling through the IL-12 domain. This fusion gene, when transfected into human HEK293 and HeLa cells, was successfully translated into mRNA, a process verified by RT-PCR. The fusion gene was also translated into protein, verified by immunohistochemistry to confirm the presence of the membrane-anchored IL-12 on the surface of transfected cells.

Future work will begin with further coculturing of the transfected human cell lines with NK cells, to measure for increased clone death and NK activation. This would be accomplished by running a cell proliferation assay on transfected clones following coculture, while NK activation would be measured by a human IFN-γ ELISA with the coculture supernatant. Following these functional assays to verify the efficacy of the fusion gene compared to the controls, further work would also entail finding a reasonable method to deliver this fusion gene to cancer cells in vivo, ideally in such a way that the delivery method can be used to treat different kinds of cancers (as this fusion gene can). Current gene therapy research still primarily aims to hone the technology to be able to target gene delivery only to specific cells, and to maintain the expression of the
introduced gene at a high enough level to achieve a therapeutic result (Liu S et al. 2014). Gene therapy as a cancer treatment could make use of viral or non-viral vectors for gene insertion, but viral vectors can be costly to manufacture and can also come with immune issues and toxicity. Non-viral vectors (be they physical, like microneedle injections or nanoparticles, or chemical, like liposomes or polymers) can be less costly to produce and less toxic than viral vectors, but their use is restricted due to low transfection efficiency or injection location (Liu S et al. 2014; Chen W et al. 2016). However, specially designed oncolytic adenoviral vectors, which do not replicate well (or at all) in normal cells but proliferate efficiently in cancer cells, may be designed to express the fusion gene (Wold WSM and Toth K, 2013). Similarly, nanoparticles may also be conjugated with, or designed to encapsulate, fusion gene DNA. Tumor specificity may be accomplished by conjugating the nanoparticles with a targeting ligand, or even simply through passively targeting tumors by taking advantage of their leaky vasculature which permits the accumulation of particles under a certain size (Lee H et al. 2016; Kim J et al. 2015).
REFERENCES


