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Killer cell activation by a novel multifunctional protein: an immuno/gene therapy for cancer

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KILLER CELL ACTIVATION BY A NOVEL MULTIFUNCTIONAL PROTEIN: AN IMMUNO/GENE THERAPY FOR CANCER

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by
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ABSTRACT

One of the characteristics of advanced tumors is the evasion of the immune system. There are multiple methods that tumor cells employ to achieve this including reducing the expression of activating ligands on the cell surface and a shift in the tumor microenvironment toward pro-tumor cytokines. The purpose of this research is to develop a novel bifunctional fusion protein that will target these two deficiencies in the tumor microenvironment and activate killer cells that are already present. The proposed protein combines the extracellular domain of a ligand for the killer cell activating receptor NKG2D and Interleukin-12 (IL-12). It is hypothesized that when expressed by tumor cells by gene therapy, the protein will simultaneously activate NK and other killer cells using the NKG2D receptor, and deliver a locally high dose of IL-12 to the tumor microenvironment where it can interact with the IL-12 receptor and enhance cytotoxicity.

We first engineered a mouse version of the protein including the extracellular domain of the NKG2D ligand mouse UL-16-binding protein-like transcript 1 (MULT1E) and mouse IL-12 (mIL-12). The fusion protein (MULT1E/mIL-12), when expressed by engineered tumor cells, indeed activated NK cells *in vitro* as assayed by increased production of interferon-γ (IFN-γ) and cytotoxicity and significantly reduced tumor growth *in vivo*.

To expand the concept of developing a novel bifunctional fusion protein for enhanced NK cell activation to human killer cells, the mouse NKG2D ligand MULT1 was replaced with the extracellular domain of a human NKG2D ligand MHC class I

polypeptide-related sequence A (MICA). The fusion protein, when expressed by engineered tumor cells, indeed activated NK92 cells as measured by an increase in IFN-γ production and an increase in cytotoxicity of tumor cells. The fusion protein was also able to increase the proliferation of human peripheral blood mononuclear cells (PBMCs) and augment their production of IFN-γ.

Although our results are preliminary, they confirm the hypothesis that a fusion protein of the extracellular domain of an NKG2D ligand and IL-12 is effective in activating killer cells *in vitro* and in animal models. The idea represents a novel immuno/gene therapy for treating human cancers.

DEDICATION

To my loving, patient, and always supportive husband.

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CHAPTER ONE

LITERATURE REVIEW

Cancer

It is projected that by 2030 there will be approximately 26 million new cancer cases and 17 million cancer deaths per year.[1] Although cancer is a heterogeneous disease with diversity not only between cancer types but also between cells within the same tumor, Hanahan and Weinberg have proposed eight hallmarks of cancer. These hallmarks describe the capabilities that normal cells acquire during tumorogenesis and include sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, deregulating cellular energetics, and avoiding immune destruction.[2]

Cancer Immunoediting

In 1909, Peter Ehrlich was the first to propose that the immune system suppressed the growth of what could be an overwhelming amount of carcinomas.[3] Because there was a lack in understanding of how this process occurred, the popularity of this theory waned. Years later, when the cellular mechanisms of immunity (including tumor specific antigens) were beginning to be elucidated, interest returned to the interactions between cancer and the immune system.[4] Burnet and Thomas authored the cancer immunosurveillance hypothesis in 1957 that stated lymphocytes recognize and eliminate

continually arising, nascent transformed cells.[4] As knowledge of the immune system has increased, it has become clear that cancer immunosurveillance is just one step of the larger immunoediting process that includes both adaptive and innate immunity.[3]

Cancer immunoediting (Figure 1.1) is a dynamic process consisting of three phases: elimination, equilibrium, and escape.[5] The elimination step includes the original idea of immunosurveillance. As the newly formed tumor begins to grow, an inflammatory response is elicited and innate immune cells (NKT, NK, and γδ T cells) are recruited to the area.[3] These immune cells are activated by a change in the structures found on the surface of transformed cells, including NKG2D ligands, and produce cytokines that aid in cell death and the recruitment of $CD4^+$ and $CD8^+$ T cells.[5] At the end of the elimination phase, any cells displaying aberrant surface structures have been removed. The equilibrium phase, which can occur over a period of several years, is sometimes referred to as dormancy. A dynamic equilibrium is reached between the rapidly mutating tumor and the host immune system following a pattern of Darwinian selection.[4] At the end of the equilibrium phase, the tumor cells that remain have been selected for their ability to evade immunosurveillance. This begins the escape phase where the selected tumors can grow in an immunologically intact environment and become clinically detectable.[3]

Tumor Microenvironment

Although cancer immunoediting can partially account for the immunoevasion seen in many cancers, it is also important to examine the immunosuppressive

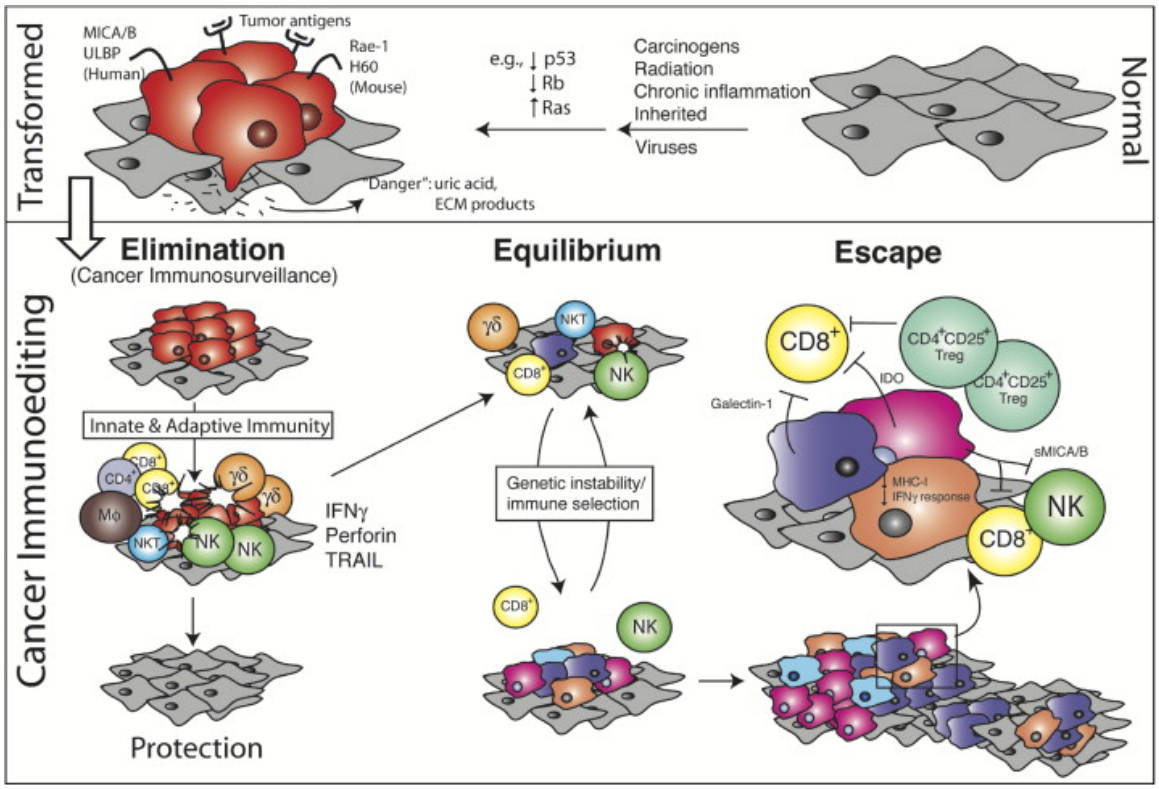

Figure 1.1 Two pathways NK cells use to cause target cell death. Exocytosis of granules containing perforin and granzymes triggers apoptotic cell death. Apoptosis can also be caused by binding of the Fas ligand to the Fas receptor of the target cell.[20]

characteristics of the tumor microenvironment. It has been demonstrated that advanced cancer patients have spontaneously generated tumor-antigen-specific immune responses such as CD8+ T cells against specific epitopes and the presence of tumor-specific antibodies. This indicates that immunologic ignorance is not to blame in all cases.[6]

There are several regulatory cell populations that are commonly found within the tumor microenvironment that function to reduce the effectiveness of activated immune cells including regulatory T cells , myeloid derived suppressor cells, and tumor associated macrophages.[6] Regulatory T cells, or Tregs, can suppress the effector function of CD4+ and CD8+ T cells, natural killer cells, natural killer T cells, B cells, macrophages, and dendritic cells. They do this by the secretion of immunosuppressive cytokines, modification or killing of antigen presenting cells (APCs), and cell-contact-dependent suppression.[7] Along with promoting tumor angiogenesis and metastasis, myeloid derived suppressor cells (MDSCs) can inhibit both adaptive and innate immunity. T cells are suppressed by the depletion of L-arginine, from the microenvironment by MDSCs that express high amounts of the enzymes arginase 1 and iNOS.[8] MDSCs can also inhibit TCR binding in T cells by both nitrating the tyrosines (through the release of ROS and peroxynitrite) and downregulating the production of the TCR ζ-chain.[9,10] Tumor associated macrophages, or TAMs are predominately polarized type II due to the increased levels of IL-10, IL-4, and IL-13 in the tumor microenvironment and promote tumor growth in several different ways. First, TAMs can promote tumor growth and survival through the production of growth factors and aid in metastasis by digesting extracellular matrix. TAMs can directly suppress T cell activation and proliferation

through the production of prostaglandins, IL-10, and indoleamine dioxigenase (IDO) metabolites and indirectly through the induction of Tregs.[11]

Tumor cells are also capable of releasing their own immunosuppressive chemicals. Some of these immunosuppressive chemicals are ligands that are normally expressed on the surface of the cell that get shed, releasing a soluble form. For example, Baltz, et al. showed that a soluble form of glucocorticoid-induced TNFR-related protein ligand (sGITRL) is released by tumor cells into the microenvironment and reduces NK cell cytotoxicity and IFN-γ production in a concentration dependent manner.[12] Other chemicals are normally produced by noncancerous cells but tumorigenesis causes these cells to produce an aberrant amount. Transforming growth factor-β (TGF-β) production is increased in many types of tumors. TGF-β reduces the effectiveness of CD4+ and CD8+ T cells, natural killer cells, dendritic cells, and type I neutrophil and macrophage development. It also induces the generation of Tregs, type II neutrophils and macrophages, and increases the immune suppression function of MDSCs.[13]

Natural Killer Cells

Human natural killer cells (NK cells) are a very important part of the innate immune system and make up approximately 15% of all circulating lymphocytes.[14,15] NK cells are granulocytes that can rapidly kill pathogen-infected cells and tumor cells but are tightly regulated by a balance of inhibitory and activating receptors.

Most inhibitory receptors are specific to MHC class I molecules that are found on every nucleated cell of the body. MHC class I molecules present cytosolic peptides on

the surface of a cell. If the peptides presented are "self," the cytolytic activity of NK cells will be inhibited. The inhibitory receptors, including killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs), contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) that, when phosphorylated, recruits phosphatases to the interface between the NK cell and its prospective target cell.[16] The tyrosine phosphatases then dephosphorylate the protein substrates of the tyrosine kinases linked to activating receptors, suppressing their activity.[17] This activity, however, is spatially regulated and only affects the activating receptors nearby, allowing the NK cell to be subsequently activated by target cells lacking ligands for the inhibitory receptors or containing many ligands for activating receptors.[17]

NK cells, unlike T and B cells, have many different activating receptors including CD16, NKp46, NKG2D, CD244, CD226, and CD2. It appears that only CD16 can be induced to cause cytotoxicity independently. All other activating receptors require synergism with other receptors to produce a cytotoxic response.[18] The variety of activating receptors induces several common signaling pathways, including ITAM/DAP12, DAP10, and ITSM, all leading to a calcium flux.[17] When an NK cell is activated, cytotoxic granules are exocytosed into the extracellular space between the NK cell and the target cell in a calcium dependent manner.[19] NK cell granules contain proteins including perforin, granzymes, and granulysin. Perforin creates transmembrane pores in the target cell and it may also be important in disrupting endosomal trafficking after it enters the cytoplasm. Granzymes work with perforin to coordinate apoptotic death of the target cell. These proteases can cleave lamins to disrupt the nuclear envelope, create single-

stranded DNA nicks, and activate caspase-mediated apoptosis. Granulysin exhibits lytic activity against both microbes and tumors.[20] Activated NK cells also have an overexpression of the Fas ligand.[21] When the Fas ligand comes in contact with the Fas expressing target cell, a death-inducing signaling complex (DISC) is formed. FADD/MORT, an adaptor, binds to Fas and engages procaspase-8. Autoproteolysis of procaspase-8 to caspase 8 occurs due to the "induced proximity" model. Caspase 8 will activate caspase-3 and caspase-7 which induce cellular apoptosis (Figure 1.2).[22]

In vitro Manipulated Natural Killer Cell Therapy

With the important role that NK cells play in recognizing and destroying tumor cells *in vivo*, they have been considered a hopeful target for cancer immunotherapy. It was first shown in the early 1980s that lymphokine-activated NK cells are able to actively clear circulating tumor cells during the first four hours after inoculation.[23] Later that decade, it was shown that NK cells can kill MHC class I deficient tumor cells that have evaded T cell cytotoxicity.[24,25] The use of autologous lymphokine-activated killer cells looked promising, but soon met difficulties because it required the co-administration of IL-2, which is very toxic at high systemic doses[26–28]. Phase II and III clinical trials also showed that there was only a 15-20% response rate which is not superior to IL-2 monotherapy or IL-2 combined with IFN-α.[29] This may be because of the presence of ligands on tumor cells for inhibitory KIRs on NK cells, resulting in the inactivation of the killer cells or an increase in the Treg cell population after IL-2 administration.[30]

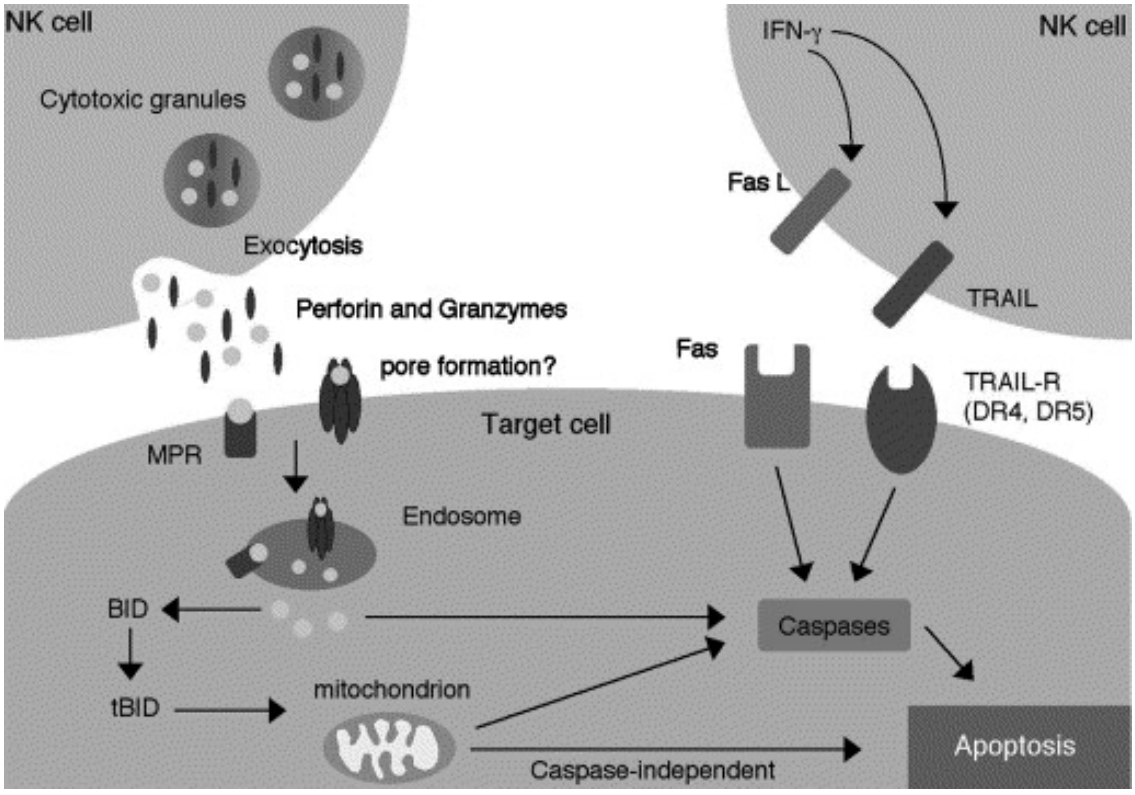

Figure 1.2 Two pathways NK cells use to cause target cell death. Exocytosis of granules containing perforin and granzymes triggers apoptotic cell death. Apoptosis can also be caused by binding of the Fas ligand to the Fas receptor of the target cell.[20]

In an effort to remedy this, NK cell donors were chosen with KIR-ligand mismatch.[29] Acute myeloid leukemia patients undergoing haploidentical stem cell transfer with KIR-ligand mismatch showed improved disease-free survival and reduced graft-versus-host disease (GVHD).[31] The clonal NK cell line, NK-92, has also been the subject of study because it lacks KIRs. The highly cytolytic NK-92 cells are activated by IL-2 and have a broad spectrum of tumor targets, however, the IL-2 activation can only be maintained *in vivo* for 48 hours.[32] In order to provide a constant supply of IL-2 for the NK cells *in vivo* and without using high doses of systemic cytokine, IL-2 secreting NK-92 clones were engineered that continually release a small amount of IL-2 allowing for prolonged activation (NK-92 MI).[33] NK-92 therapy has been shown to be effective for leukemia and trastuzumab resistant breast tumor cells *in vitro*, and malignant melanoma and renal cell carcinoma *in vivo*.[33–36] These treatments have also been more effective than lymphokine-activated killer cells.[33]

NKG2D: A Natural Killer Cell Activating Receptor

Another approach is activating NK cells *in vivo* through activating receptors. The activating receptor NKG2D is found as a homodimer on NK cells, γδT cells, some $CD8^+$ αβT cells, NKT cells, and a subset of $CD4^+$ αβ T cells and plays an important role in cancer immunity (Table 1.1).[37–39]

NKG2D ligands include MHC class I related chain (MIC) A and B and the UL-16 binding protein family in humans and RAE-1, H-60, and mouse UL-16 binding protein-like transcript (MULT1) in mice.[40] The transmembrane domain of NKG2D associates

Table 1.1 Expression of NKG2D by immune cells. There is a differential expression of the activating receptor NKG2D on mouse and human cells.[39]

Table 1 | **Expression of NKG2D by immune cells**

Cell type	Cell-surface expression pattern	
	Mouse	**Human**
NK cells	~100%	~100%
$CD8^+$ αβ T cells	Before activation: absent After activation: ~100% Antigen-specific memory cells:~100%	Before activation: ~100% After activation: ~100% Antigen-specific memory cells: ~100%
$CD4^+$ αβ T cells (conventional)	Rare or absent	Normally absent, upregulated in rheumatoid arthritis patients
$NK1.1^+$ T cells	~70% positive	N. D.
γδ T cells	Splenic γδ T cells: ~25% Intestinal intraepithelial γδ cells: absent Dendritic epidermal γδ T cells: ~100%	Peripheral blood γδ T cells: ~100% Intestinal intraepithelial γδ cells: ~100%
Macrophages	Before activation: absent After activation (with LPS, IFN-α/β or IFN-γ): ~100%	Absent on monocytes and macrophages

IFN, interferon; LPS lipopolysaccharide; N. D. not determined; NK, natural killer.

with the transmembrane domain of the DAP10 homodimer in humans forming a hexadimer receptor complex with one NKG2D homodimer and two DAP10 homodimers. In mice, two forms of NKG2D exist: NKG2D-L and NKG2D-S depending on the number of amino acids in the cytoplasmic domain. NKG2D-L can only pair with DAP10. However, NKG2D-S can pair with either DAP10 or DAP12.[17]

When DAP10 is phosphorylated, signaling can occur through the p85 subunit of phosphatidylinositol-3-OH kinase (PI3K) or the adaptor Grb2 binding to the YINM signaling motif. Both p85 and Grb2 binding lead to a Ca^{2+} influx, NKG2D mediated cytotoxicity, actin cytoskeleton reorganization, and microtubule polarization. Binding of p85 to DAP10 also induces production of phosphatidylinositol-3,4,5-triphosphate (PIP_3) in the immune synapse between the activated NK cell and the target cell.[17]

DAP12 contains an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domain which, when phosphorylated, binds the tyrosine kinases Syk or ZAP-70 (Figure 1.3). Downstream results of phosphorylation include actin cytoskeleton reorganization, release of cytolytic granules, and the transcription of cytokine and chemokine genes. Because of species specific expression of DAP10 and DAP12, mouse NKG2D ligand produce both cytotoxicity and cytokine release whereas human NKG2D ligands preferentially produces cytotoxicity.[17]

Mouse UL-16 Binding Protein-Like Transcript 1 (MULT1)

MULT1 is an NKG2D ligand with a protein sequence similar to human UL16-binding protein 3 (ULBP3).[41] The 37.1 KD protein contains two MHC class I-like α

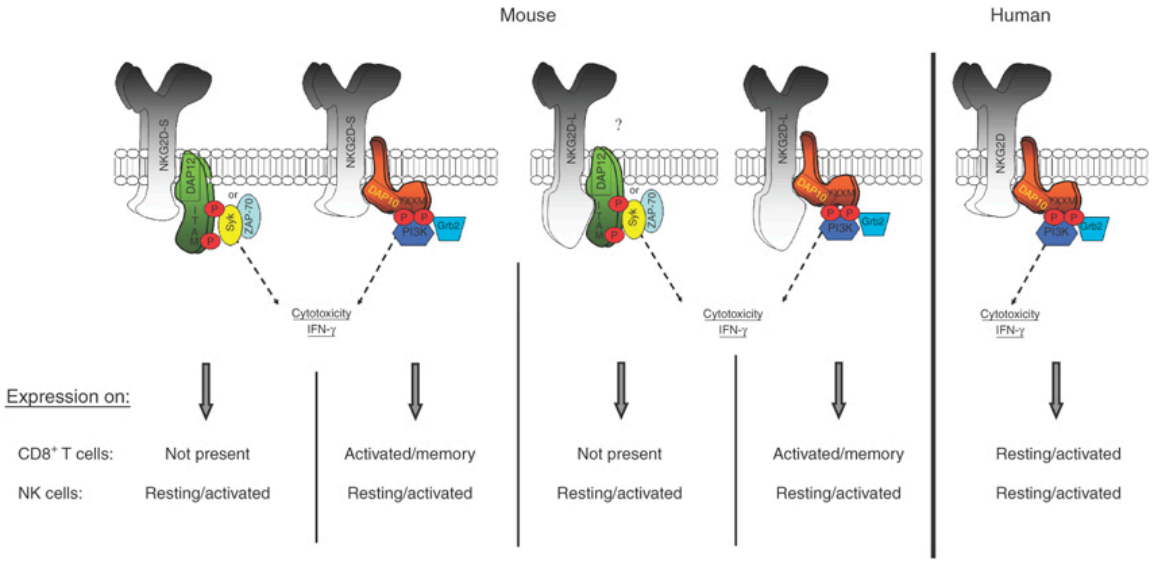

Figure 1.3 NKG2D Receptor. The mouse has both NKG2D-S and NKG2D-L receptors. The NKG2D-S can pair with either DAP12 or DAP10. NKG2D-L can pair with DAP10. The one form of human NKG2D only pairs with DAP10.[111]

domains.[42] Unlike ULBP or the RAE1 family proteins that are anchored to the cell membrane by glycosylphosphatidylinositol (GPI), MULT1 is a type I transmembrane protein with both a transmembrane and a cytoplasmic domain.[42] MULT1 transcripts are detectable in many normal tissues including thymus, spleen, lymph nodes, liver, heart, and lung, unlike other mouse ligands for NKG2D.[42] However, no surface expression is detected when stained with NKG2D tetramers suggesting that MULT1 is regulated post-transcriptionally. Nice et al. showed that MULT1 is regulated posttranslationally through ubiquitination dependent on lysines in its cytoplasmic tail and lysosmal degradation under normal conditions.[43] Degradation and uniquitination of MULT1 can be decreased by heat shock or ultraviolet irradiation leading to higher surface expression. The same results were not seen with ionizing radiation, 5-FU, cisplatin, or mitomycin C.[43]

MHC Class I Chain-Related Protein A (MICA)

MHC class I Chain-Related Protein A (MICA) is a human NKG2D ligand. Unlike MULT1, under normal conditions, the mRNA for this nonconventional HLA class I molecule is expressed only in intestinal and thymic epithelium.[44,45] However, under stress (like tumorigenesis and exposure to intracellular pathogens), many epithelial cells can upregulate MICA expression.[46] The highly polymorphic MICA gene codes for a membrane-bound protein made of 383 amino acids and a relative molecular mass of 43kDa.[47] Although MICA conserves the α1-3 extracellular domains seen in classical MHC class I molecules, the MHC peptide-binding groove is not open to allow peptide presentation.[39]

There has been a correlation found between the presence of soluble MICA in the sera of cancer patients and cancer stage and metastasis.[48] This correlation is commonly attributed to downregulation of NKG2D receptor expression on effector cells.[49] However, recent studies have shown that the NKG2D receptor downregulation may be mediated instead by other factors including TGF-β and membrane bound MICA. It has also been shown that the downregulation of NKG2D receptor caused by soluble MICA is at supraphysiological levels.[50]

Cancer Immunoediting Affects NKG2D Ligands

NKG2D ligands play an important role in the first stage of immunoediting. During the early stages of cancer development, DNA damage can cause the upregulation of NKG2D ligands, allowing the cells to be easily recognized and removed by NK cells (Table 1.2).[40] B6 and BALB/c mice treated with anti-NKG2D mAb showed an increase in sarcoma incidence after exposure to methylcholanthrene (MCA) indicating that functional NKG2D reduced sarcoma incidence.[51] As the cancer progresses, however, tumor cells have found ways to downregulate surface NKG2D ligands. It has been shown that the extracellular domains of MICA can be shed by tumor cells using proteolytic cleavages, thus preventing activation of cytotoxic immune cells.[52] The immune system may also aid in selecting against tumor cells that maintain NKG2D ligand expression.

Table 1.2 Induction of NKG2D ligands by stress pathways. NKG2D ligand expression is upregulated in response to stress within the cell. Different stress pathways cause upregulation of different ligands.[112]

Refs refer to references in original paper.

Level of regulation	Underlying pathway	Ligands regulated	Regulatory components	Refs
mRNA (transcription or mRNA stabilization)	Heat shock response	MICA and MICB (in humans)	Heat shock transcription factors	96
mRNA (transcription or mRNA stabilization)	DNA damage response	ULBP, MICA (in humans); RAET1, MULT1 and H60a (in mice)	ATR-, ATM- and CHK-dependent; p53 not required	90
mRNA (transcription or mRNA stabilization)	Cell senescence	MICA, MICB and ULBP2 (in humans)	ATR- and ATM-dependent in some cases	57,93
mRNA (transcription or mRNA stabilization)	Wounding	H60c (in mice)	ND	105
Protein stabilization	Heat shock response; UV irradiation	MULT1 (in mice)	Independent of DNA damage response	97

ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3 related; CHK, checkpoint kinase; H60, histocompatibility 60; MICA, MHC class I polypeptide-related sequence A; MULT1, murine ULBP-like transcript 1; ND, not determined; RAET1, retinoic acid early transcript 1; ULBP, cytomegalovirus UL16-binding protein; UV, ultraviolet.

NKG2D Ligand Immunotherapy

It should be possible, then, to reinitiate an immune response by upregulating NKG2D ligands in cancer cells. In 2001, groups at both the University of California, Berkeley and the University of California, San Francisco concurrently, but separately, showed that by transfecting several tumor cell lines to express Rae1 or H60 using a retroviral vector, tumor growth could be reduced or abrogated completely.[53,54] The Berkeley group showed that along with activating NK cells, $CD8^+$ T cells were also stimulated by some of the tumor cell lines leading to a specific immune memory response even against the parental non-transfected tumor cells.[53] However, the group from San Francisco was not able to show the same response with Rae1 transfected RMA lymphoma tumors.[54] Ectopic expression of ULBP1, ULBP2, or ULBP3 has also been shown to cause reduced tumor growth.[55] Others have shown that upregulating MICA on glioma and melanoma cells promotes NK-mediated cytolysis and induces T cell response.[56,57]

NKG2D ligands have also been used in modified forms. For example, a fusion protein of MULT1 and Fas has been shown to significantly decrease tumor cell growth in mouse models. The extracellular domain of the MULT1 NKG2D ligand functions as an activator of NK cells in the environment whereas the transmembrane and intracellular domains of Fas lead to Fas-mediated apoptosis. The addition of Fas increased the tumor suppressing abilities of the MULT1.[58] Additionally, the therapeutic value of this fusion protein was affirmed by the production of a replication-deficient adenovirus vector that,

when injected into a subcutaneous tumor, caused a significant decrease in tumor growth.[59]

Another approach is to generate a bispecific secreted protein where the NKG2D ligand domain is conjugated to an antibody to direct the protein to the tumor cell surface. The bispecific protein ULBP2-BB4 binds both to CD138, which is overexpressed on the surface of a variety of malignant cells, through the BB4 moiety and to NK cells using the ULBP2 moiety.[60] The combination therapy of ULBP2-BB4 protein and peripheral blood lymphocytes (PBLs) was highly effective in eradicating tumors in a multiple myeloma xenograft model.[60] By using a purified protein, there is no need for the sometimes controversial gene delivery.

Interleukin 12

Interleukin 12 (IL-12), also known as NK cell stimulatory factor 2, is also an activator and initiator of NK cell proliferation. It is a heterodimeric cytokine consisting of the disulfide-linked subunits p35 and p40 (named according to their molecular weight).[61] The IL-12 receptor is composed of two subunits: IL-12Rβ1 and IL-12Rβ2 and are found on activated T, NK and NKT cells.[62,63] IL-12Rβ1 and IL-12Rβ2 occur on the cell surface as oligomers. Oligomerization of the subunits is ligand independent.[62] IL-12Rβ1 binds the IL-12 p40 subunit whereas IL-12Rβ2 recognizes either the heterodimer IL-12 or the IL-12 p35 subunit. Upon ligand binding, activation of Jak kinases occurs. Tyk-2 is associated with IL-12Rβ1 and Jak-2 is associated with IL-12Rβ2 causing phosphorylation of the IL-12 receptor. The phosphorylated receptor then phosphorylates

STAT4 proteins. These phosphorylated STAT4 proteins then form homodimers and translocate to the nucleus where they function as transcription factors (Figure 1.4).

IL-12 functions as a mediator between innate and adaptive immunity. At the onset of an infection, IL-12, produced by activated macrophages, enhances the cytotoxicity and proliferation of NK and T cells.[64] IL-12 stimulates NK cytolytic activity against NK-sensitive targets, NK-resistant targets, and antibody-coated targets. This activity is more efficient than either IL-2 or the IFNs; requiring picomolar instead of nanomolar concentrations.[65] In developing Th1 cells, IL-12 leads to the upregulation of IL-18 and adhesion molecules such as P- and E- selectin ligands allowing them to be easily recruited to sites where an immune response is needed.[66] IL-12 has been shown consistently to have potent anti-tumor activity primarily through the activity of IFN-γ, the activation of $CD8^+$ T cells, and the inhibition of angiogenesis.[67,68] In fact, it is thought that IL-12 is responsible for the anticancer effects of Coley's toxin, a mixture consisting of killed bacterial species. In the treatment of inoperable sarcoma, Coley's toxin provided a 50% complete tumor regression at the time of therapy and more than 5-year survival. Twenty percent of patients were cured and survived over 20 years. Although Coley's toxin was discovered over 100 years ago, modern immunotherapy trials have yet to surpass these survival rates. Coley's toxin can kill both developed tumors, through T cells, and newly established tumors, through NK cells.[69]

Interferon-γ

IL-12 also works in conjunction with IL-2 as a potent inducer of several cytokines

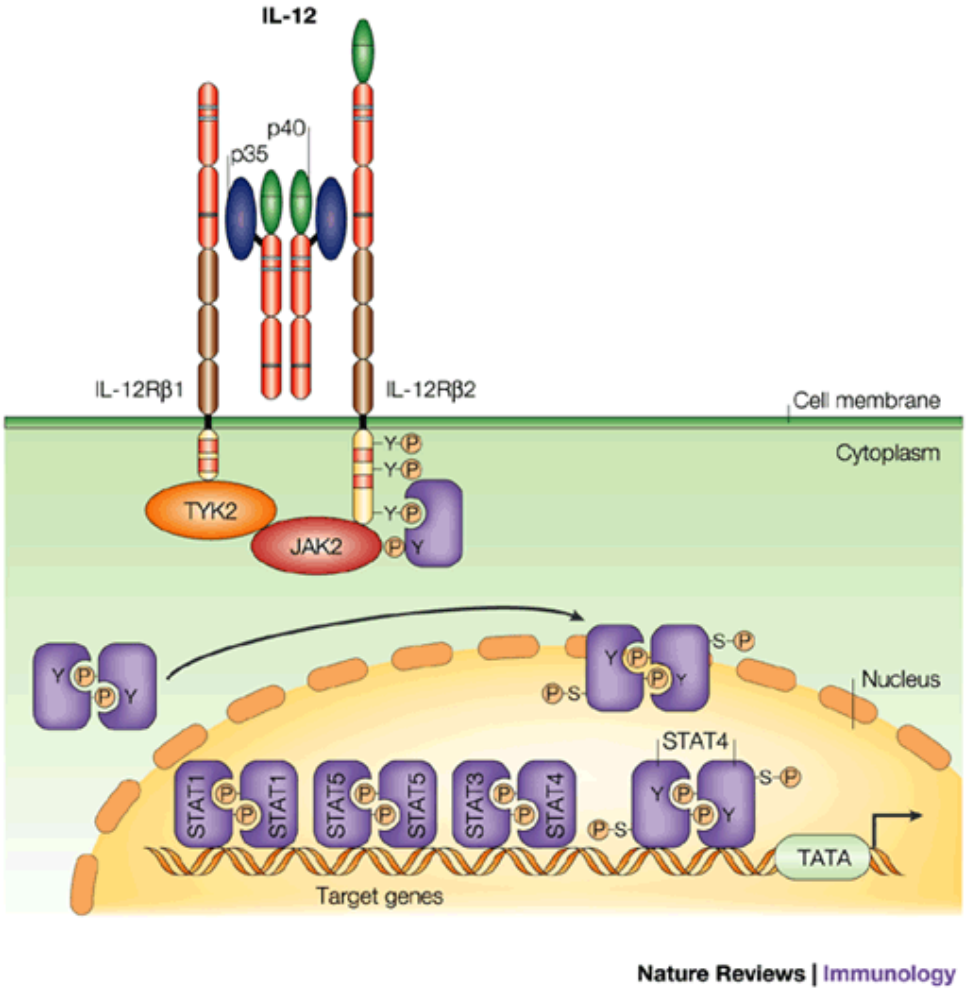

Figure 1.4 IL-12 Receptor. The IL-12 receptor uses a Jak/STAT signaling pathway.[113]

but most importantly Interferon-γ (IFN-γ). IL-12 alone has been shown to increase the production of the IFN-γ transcripts whereas IL-12 and IL-2 together increase IFN-γ mRNA half-life.[70] T lymphocytes and NK cells produce IFN-γ. At physiologic conditions, IFN-γ is found as a homodimer held together by noncovalent bonds.[71] The IFN-γ receptor is ubiquitously expressed on all cells except erythrocytes and is made up of two subunits: the high affinity receptor (IFN-γR1) and a low affinity receptor (IFN-γR2).[71,72] The IFN-γ homodimer binds to two IFN-γR1 chains that are preassembled with IFN-γR2.[73] The resulting receptor complex leads to transcriptional activity predominantly through the JAK/ STAT pathway (Figure 1.5).[74]

IFN-γ has many important biological functions due to its control over a large number of genes. IFN-γ is an important component of the cytokine cocktail that promotes the development of helper T cells into Th1 cells while inhibiting the development of Th2 cells, initiating cell-mediated immunity through the activation of $CD8^+$ T cells.[75] B cells are also affected with an increase in isotype switching to IgG2a.[76] IFN-γ increases monocyte microbicidal functions including secretion of hydrogen peroxide, NO production, tryptophan depletion, and upregulation of lysosomal enzymes.[77,78] NO production also leads to local dilation of blood vessels causing gathering of blood and eventually extravasation.[79] It can also induce expression of angiogenesis inhibitors, including IP10, that limit the size of tumor growth.[80] It can alter the proteasome to add variety and increase peptides available for MHC class I expression. In addition, IFN-γ can increase the availability of the TAP transporter that helps provide efficient peptide loading.[79] IFN-γ can also arrest the cell cycle usually at the G1/S

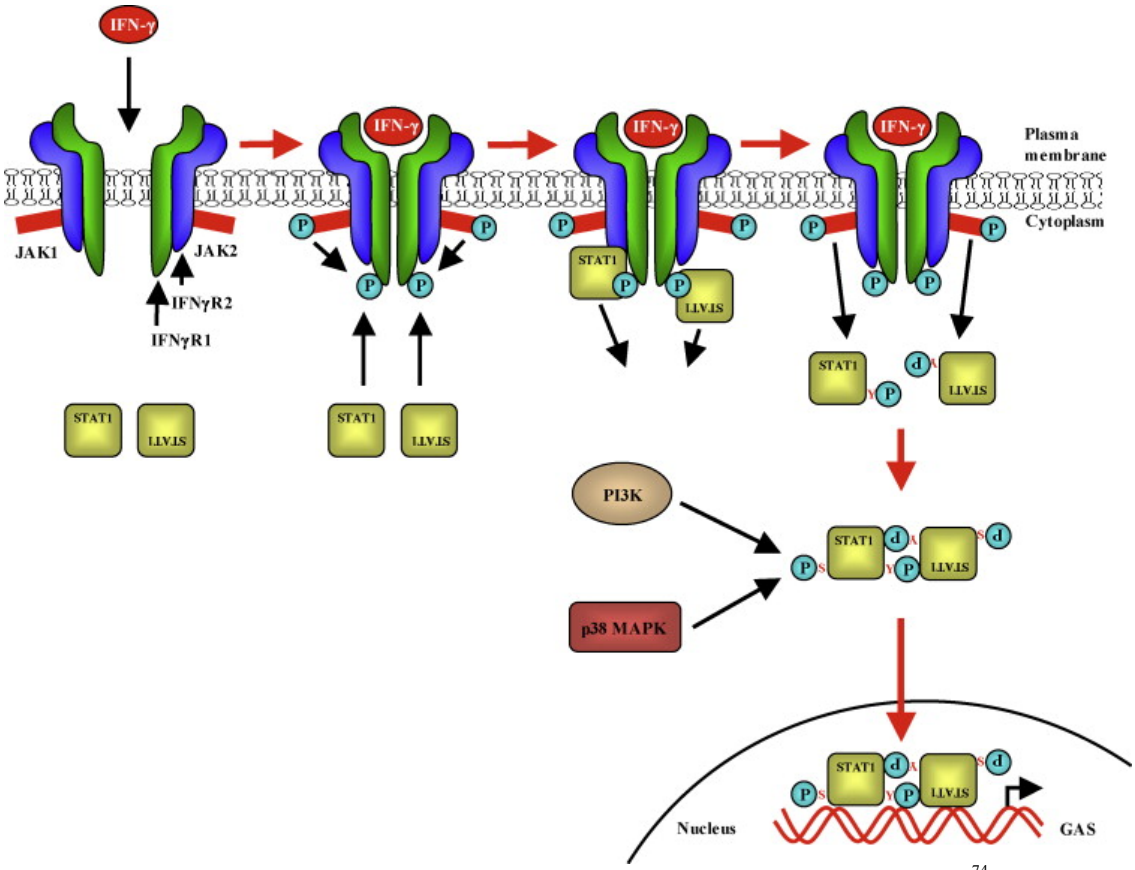

Figure 1.5 IFN- γ signal transduction. IFN-γ receptor is part of a Jak/STAT signaling cascade.[74]

checkpoint. This is at least partially accomplished by inducing the transcription of p21 and p27 cyclin kinase inhibitors.[81] Depending on the cell surface density of IFN-γR2, apoptosis can be induced. In cells with high surface levels of IFN-γR2, there is rapid activation of STAT1 and high levels of interferon regulatory factor-1 (IRF-1), which triggers apoptosis through caspase 1.[82]

Cytokine Therapy

Cytokines are immunomodulatory molecules that play significant roles in coordinating immune responses by signals through autocrine, paracrine, or endocrine means. By increasing stimulatory cytokines, it may be possible to manipulate an immune response. Many cytokines have been tested for anticancer abilities with the majority proving to be toxic or ineffective. Interleukin 2 (IL-2) and interferon-alpha (IFN-α) have been approved by the FDA to treat kidney cancer, leukemias, lymphomas, myelomas, and melanoma.[83] These drugs, however, do not specifically initiate an immune response against tumor cells. For this reason, a large dose is required to get a clinically relevant response. Many patients experience toxicity with high systemic doses of cytokines. To ameliorate the requirement for high doses, cytokines have been engineered to specifically target the tumor microenvironment.

Several methods have been employed to target IL-2 and IFN-α to tumor cells using antibodies. By linking IL-2 to L19, an antibody specific to tumor neovasculature, tumor size can be reduced significantly when compared to non-linked IL-2 and gemcitabine, a first-line treatment for pancreatic cancer. This reduction was due in part

to a 70 fold increase in NK cells to the tumor microenvironment.[84] Similar results are found when L19 is linked to IL-12 or TNF-α.[85] IL-2 has also been linked to single-chain T cell receptor that recognizes the aa264-272 peptide of human p53. p53, which is found mutated in approximately half of all cancers, is not expressed on the surface of cells. Peptides can, however, be displayed in the context of MHC class I molecules to be recognized by T cells. This fusion protein significantly reduced A375 melanoma tumor growth in nude mice.[86]

Another approach is to anchor cytokines to the surface of cancer cells by adding glycosylphosphatidylinoditol (GPI). B16F0 melanoma cells transfected with GPI-IL-2, when delivered intravenously to C57BL/6J mice, grew significantly slower than B16F0 cells and B16F0 cells transfected to secrete IL-2. The cells containing GPI-IL-2 were also effective in preventing the growth of nearby B16F0 cells not expressing IL-2.[87] Similar results were seen with a GPI-IL-12 construct. When GPI-IL-2 and GPI-IL-12 were coexpressed on the B16F0 cells, the survival of the tumor bearing animals was significantly improved.[88] Melanoma antigen-1 (MAGE-1)-expressing T24 tumor cells have also been transfected with GPI-IL-2 for the production of tumor-derived exosomes (TEXs). Preliminary *in vitro* studies show that dendritic cells pulsed with these TEXs induce MAGE-1-specific CTLs more efficiently than dendritic cells pulsed with MAGE-1 TEXs without GPI-IL-2.[89]

All of the above methods involve anchoring the cytokine in the tumor microenvironment either through ligand interaction or GPI anchoring on the cell surface.

Another method to reduce the amount of systemic cytokine required is to increase the binding affinity of the cytokine to its receptor. The IL-2 high affinity quaternary receptor complex is composed of IL-2, IL-2Rα (CD25), IL-2Rβ, and IL-2Rγ. All three receptors are found in low densities on resting T cells making them relatively insensitive to IL-2 requiring high doses to initiate a response. When T cells are activated through the T cell receptor, CD25 is upregulated.[90] CD25 can then capture the IL-2 and present it to IL-2Rβ and IL-2Rγ forming a high affinity receptor. Levin et al have used *in vitro* evolution to produce an IL-2 "superkine" (also called super-2) that does not require CD25. The IL-2 superkine has a higher binding affinity for IL-2Rβ due to a reduction in flexibility of a helix in the IL-2Rβ binding site; this is similar to the conformational state found when IL-2 is bound to CD25. The T cell expansion initiated by the IL-2 superkine is similar to IL-2 but can progress at a much lower concentration reducing toxicity.[91]

NKG2D Ligands and IL-12 Function Together

IL-12 has been shown to be an effective preventative treatment for methylcholanthrene (MCA) induced sarcoma with its effectiveness largely due to NKG2D/NKG2D ligand binding. When mice were given IL-12 treatments prior to a lethal dose of MCA, the mice were protected. However, when the IL-12 treatment was given along with an anti-NKG2D mAb the protective effects were diminished significantly.[51] NKG2D engagement along with IL-12 therapy also causes an increase in cytotoxicity and IFN-γ production *in vitro*.[92] This could be caused by an increase in expression of NKG2D ligands, TRAIL and perforin transcripts, and increased

phosphorylation of STAT1, STAT4, and ERK1/2 in NK cells activated with IL-12 and NKG2D engagement.[93] Along with native NKG2D expression, the introduction of soluble ULBP with IL-12 stimulated production of GM-CSF, TNF-β, and the CC chemokine I-309 by NK cells *in vitro* whereas the use of either alone showed little effects on these cytokines.[94] This combination of IL-12 and NKG2D activity appears to be an effective method for NK cell activation and target cell cytotoxicity.

CHAPTER TWO

INTRODUCTION AND SPECIFIC AIMS

We hypothesize that a fusion protein containing an NKG2D ligand and IL-12 when secreted from tumor cells into the tumor microenvironment will synergize the activation of killer cells. The NKG2D ligand will perform two functions. First, it will activate killer cells in the area using the NKG2D receptor causing an increase in killer cell mediated cytotoxicity. Secondly, the NKG2D ligand will function as a carrier to deliver IL-12 directly to the killer cells. When the NKG2D ligand binds to an NKG2D receptor on the surface of a killer cell, the IL-12 will be held in close proximity to IL-12 receptors on the same cell. The IL-12 will then also activate the killer cell leading to cytotoxicity and proliferation. This dual activation of killer cells in the tumor microenvironment should lead to increased tumor cell death and decrease in tumor volume (Figure 2.1).

Compared to traditional chemotherapy and radiation treatments, this novel fusion protein has several benefits. This therapy takes advantage of the body's natural defense mechanisms that are already in place. Because this novel fusion protein activates killer cells that are part of the innate immune system that does not require the use of tumor specific antigens, this therapy should be easily translatable to many different tumor types. Using gene therapy to deliver the fusion protein directly to the tumor cells will maintain a high level of IL-12 in the tumor microenvironment while providing low systemic IL-12 levels, reducing toxicity.

In our previous studies, we created a fusion gene consisting of the extracellular domain of MULT1 and the transmembrane and intercellular domains of mouse Fas and demonstrated that fusion protein MULT1E/mFasTI activates NK cells and induces apoptosis of tumor cells expressing the protein *in vitro* and *in vivo*.[59] The limitation of fusion protein MULT1E/mFasTI is that it can only activate the NK cells that are engaged with the tumor cells expressing the protein and it cannot activate other NK cells in the tumor environment. The bifunctional protein created in this study, however, can provide a local high dose of IL-12, which is able to activate NK cells in the local tumor environment so that the therapy may be more effective.

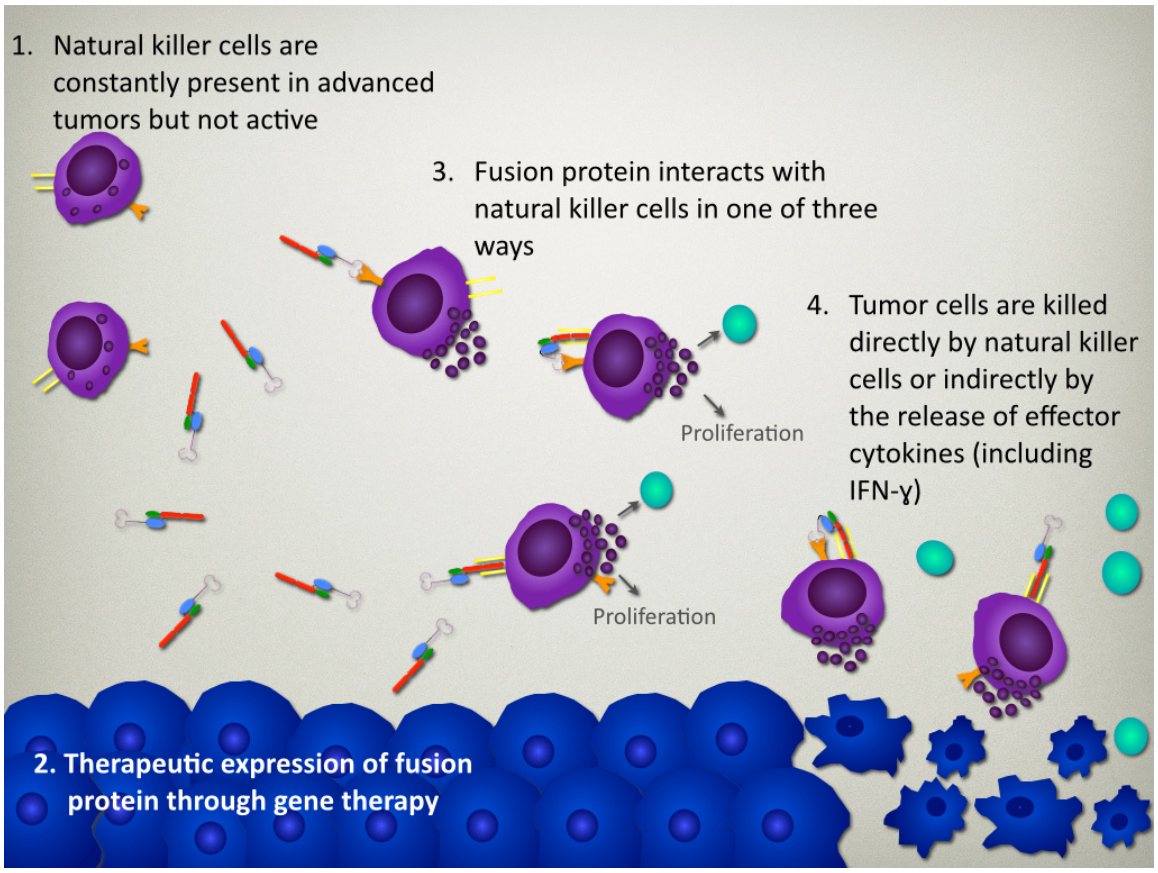

Figure 2.1 Summary of hypothesis

We will test our hypothesis by pursuing the following specific aims:

1. ***The construction of an expression vector containing the MULT1E/mIL-12 fusion gene*** The extracellular domain of MULT1 and the entire mIL-12 gene will be cloned into the pcDNA3.1(+) expression vector. Expression vectors will also be constructed to serve as controls: one with MULT1E alone and another with mIL-12 alone.

2. ***Transfection and selection of stable clones expressing the MULT1E/mIL-12 fusion protein*** The pcDNA3.1(+) expression vectors will be used to transfect TC-1 mouse lung carcinoma tumor cells using Lipofectamine. Stable clones will be selected and characterized using RT-PCR, qRT-PCR, fluorescent microscopy, immunofluorescence, and ELISA.

3. **In vitro *biological activity of the MULT1E/mIL-12 fusion protein*** Mouse NK cells will be purified using negative selection from spleen cells with a MACS mouse NK cell isolation kit. These NK cells will be co-cultured with clones expressing the fusion protein. When NK cells are activated, they release a cocktail of cytokines including IFN-γ. The amount of IFN-γ produced will be evaluated with ELISA to determine the level of activation. To determine if there is a reduction in tumor cells due to NK cytotoxicity, Promega's CellTiter 96 AQ_{ueous} will be used.

4. **In vivo *antitumor activity of MULT1E/mIL-12 fusion protein secreted by tumor cells*** C57BL/6 mice will be injected intravenously with transfected tumor cells expressing the MULT1E/mIL-12 fusion protein. After four weeks, tumor nodules

will be counted and the weight of the lungs will be compared to determine if the fusion protein has an effect on tumor volume.

5. ***The construction of an expression vector containing the MICA/IL-12 fusion gene*** The extracellular domain of MICA and the entire IL-12 gene will be cloned into the pcDNA3.1(+) expression vector. Expression vectors will also be constructed to serve as controls: one with MICA alone and another with IL-12 alone.

6. ***Transfection and selection of stable clones expressing the MICA/IL-12 fusion protein*** The pcDNA3.1(+) expression vectors will be used to transfect A549 human lung carcinoma tumor cells using Lipofectamine. Stable clones will be selected and characterized using RT-PCR, immunofluorescence, and ELISA.

7. **In vitro *biological activity of the MICA/IL-12 fusion protein*** The human NK cell line NK92 will be used to analyze the activating ability of the MICA/IL-12 fusion protein. These NK92 cells will be co-cultured with clones expressing the fusion protein. When NK92 cells are activated, they release a cocktail of cytokines including IFN-γ. The amount of IFN-γ produced will be evaluated with ELISA to determine the level of activation. To determine if there is a reduction in tumor cells due to NK cytotoxicity, Promega's CellTiter 96 AQ_{ueous} will be used. NK92 cells will also be primed with supernatant collected from the A549 clones and co-cultured with parental A549 cells to determine if priming can increase the killing of cells that do not produce the protein. Lastly, human PBMCs will be isolated from leukapheresis product using the Ficoll-Paque method and supernatant

collected from the A549 clones will be used to activate them. Activation will be determined using an IFN-γ ELISA. Promega's CellTiter 96 AQ_{ueous} will also be used to determine if the protein can increase PBMC proliferation.

CHAPTER THREE

MULT1E/mIL-12: A NOVEL BIFUNCTIONAL PROTEIN FOR NATURAL KILLER CELL ACTIVATION[1]

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Abstract

Natural killer (NK) cells have the potential to be effective killers of tumor cells. They are governed by inhibitory and activating receptors like NKG2D, whose ligands are normally upregulated in cells that are stressed, like cancer cells. Advanced cancer cells, however, have ways to reduce these ligands' expression, leaving them less detectable by NK cells. Along with these receptors, NK cells also require activating cytokines, like interleukin 12 (IL-12). The goal of this study is to develop a novel bifunctional fusion protein for enhanced NK cell activation. The proposed protein combines the extracellular domain of the NKG2D ligand Mouse UL-16-binding protein-like transcript 1 (MULT1E) and mouse IL-12 (mIL-12). It is hypothesized that when expressed by tumor cells, the protein will activate NK and other killer cells using the NKG2D receptor, and deliver

[1] Cited from: *Gene Ther.* **21,** 468–475 (2014) with modifications

mIL-12 to the NK cells where it can interact with the IL-12R and enhance cytotoxicity. The fusion protein, when expressed by engineered tumor cells, indeed activated NK cells *in vitro* as assayed by increased production of interferon-γ and cytotoxicity and significantly reduced tumor growth *in vivo*. Although the study is preliminary, the data suggest that the MULT1E/mIL-12 bifunctional fusion protein is an effective activator of NK cells for cancer treatment.

Keywords: NK cells, NKG2D, IL-12, MULT, cancer, fusion protein

Introduction

Natural killer (NK) cells are part of the innate immune system and are one of the primary cell types involved in immunosurveillance. At the onset of neoplastic growth, damaged cells upregulate surface antigens, some of which are ligands for NK cell activating receptors, including NKG2D ligands. Once NK cells are activated, cytotoxic granules are exocytosed into the extracellular space between the NK cell and the target cell.[19] As the cancer progresses, however, tumor cells have found ways to downregulate surface NKG2D ligands. It has been shown that the extracellular domains of MHC class-I related molecule A (MICA), a human NKG2D ligand, can be shed by tumor cells by proteolytic cleavages, thus preventing activation of cytotoxic immune cells.[52] The immune system may also aid in selecting against tumor cells that maintain NKG2D ligand expression.

Mouse UL-16 binding protein-like transcript 1 (MULT1) is an NKG2D ligand with a protein sequence similar to human UL16-binding protein 3.[41] The 37.1 KD protein contains two major histocompatibility class I-like α domains. Unlike ULBP or the RAE1 family proteins that are anchored to the cell membrane by glycosylphosphatidylinositol, MULT1 is a type I transmembrane protein with both a transmembrane and a cytoplasmic domain. Unlike other mouse ligands for NKG2D, which are expressed and only upregulated on diseased cells, MULT1 transcripts are detectable in many normal tissues including thymus, spleen, lymph nodes, liver, heart, and lung.[42] No surface expression of MULT1 is detected, however, when stained with

NKG2D tetramers suggesting that MULT1 is regulated post-transcriptionally. Nice et al showed that MULT1 is regulated posttranslationally through ubiquitination dependent on lysines in its cytoplasmic tail and lysosmal degradation under normal conditions.[43] Degradation and ubiquitination of MULT1 can be decreased by heat shock or ultraviolet irradiation leading to higher surface expression. The same results were not seen with ionizing radiation, 5-FU, cisplatin, or mitomycin C.[43] Due to these properties, MULT1 serves as an excellent target of anti-cancer immunotherapies.

Interleukin 12 (IL-12), also known as NK cell stimulatory factor 2, is an activator and initiator of NK cell proliferation. This activity is more efficient than either IL-2 or the interferons (IFNs) requiring picomolar instead of nanomolar concentrations.[65] In fact, NK cells expressing an IL-12 and mouse sonic hedgehog C-terminal domain fusion gene showed reduced (>10 fold) dependency on IL-2 and a significantly prolonged survival time for tumor bearing mice receiving an intravenous injection of these cells.[95] IL-12 has been shown consistently to have potent anti-tumor activity primarily through the activity of IFN-γ, the activation of $CD8^+$ T cells, and the inhibition of angiogenesis.[67,68]

We hypothesize that a bifunctional fusion protein containing the NKG2D ligand MULT1 and mouse IL-12 (mIL-12) will synergize the activation of NK cells and other NKG2D-expressing killer cells. The proposed fusion protein combines the extracellular domain of MULT1 (MULT1E) and mIL-12. When delivered to the tumor microenvironment, NK cells and other NKG2D-expressing killer cells will be activated simultaneously through the MULT1/NKG2D pathway and the IL-12/IL-12R pathway. In addition, MULT1E will also function as a carrier to deliver mIL-12 directly to the killer

cells. When the MULT1E domain binds to an NKG2D receptor on the surface of an NK cell, the mIL-12 will be held in close proximity to IL-12 receptors on the same cell, which will maintain a high level of mIL-12 in the tumor microenvironment while providing low systemic mIL-12 levels and reduced toxicity

The hypotheses were first examined by transfecting the fusion gene into the mouse lung carcinoma cell line TC-1. Stable clones of tumor cells expressing the fusion protein and control IL-12 or MULT1E proteins were evaluated in a series of *in vitro* assays. Preliminary *in vivo* studies showed that the dual activation of NK cells by the fusion protein in the tumor microenvironment led to increased tumor cell death and a decrease in tumor volume.

Results

Transfection and selection of stable clones expressing the MULT1E/mIL-12 fusion protein

TC-1 cells were transfected with pCDNA3.1(+)MULT1E/mIL-12/Zeo (Figure 3.1a) using Lipofectamine. Clones that were zeocin resistant were selected and labeled as M-IL/4, M-IL/5 and M-IL/8. TC-1 cells were also transfected with pcDNA3.1(+)MULT1E/Zeo or pcDNA3.1(+)mIL-12/Zeo to generate control clones (MULT/6 and IL/3) that produce either MULT1E or mIL-12, respectively. An *in vitro* cell growth study shows that all clones grow at a similar rate (data not shown).

The fusion protein expression was first examined by reverse transcription-PCR (RT-PCR) on total RNA samples collected from the clones using three pairs of primers. The first pair was specific to a 1.6-kb portion of the MULT1E/mIL-12 fusion gene containing 57 bp of MULT1E, the (GGGS)3 linker and the 1545 bp of mIL-12. The second pair amplified a 439-bp sequence near the 3′ end of mIL-12. The third pair amplified the entire 633 bp MULT1E sequence. Only clones M-IL/4, M-IL/5 and M-IL/8 contain mRNA transcripts for the MULT1E/mIL-12 fusion gene (Figure 3.1b). As expected, the fusion gene clones, along with the IL/3 clone contain mRNA transcripts for the 3′ segment of mIL-12 (Figure 3.1c). All cells, including the TC-1 control cells and IL/3, contain mRNA transcripts for MULT1E (Figure 3.1d). Although quantification is not accurate with RT-PCR, the densitometry measurements show that Clone M-IL/4 produced more transcripts of the fusion gene than Clones M-IL/5, which produced more

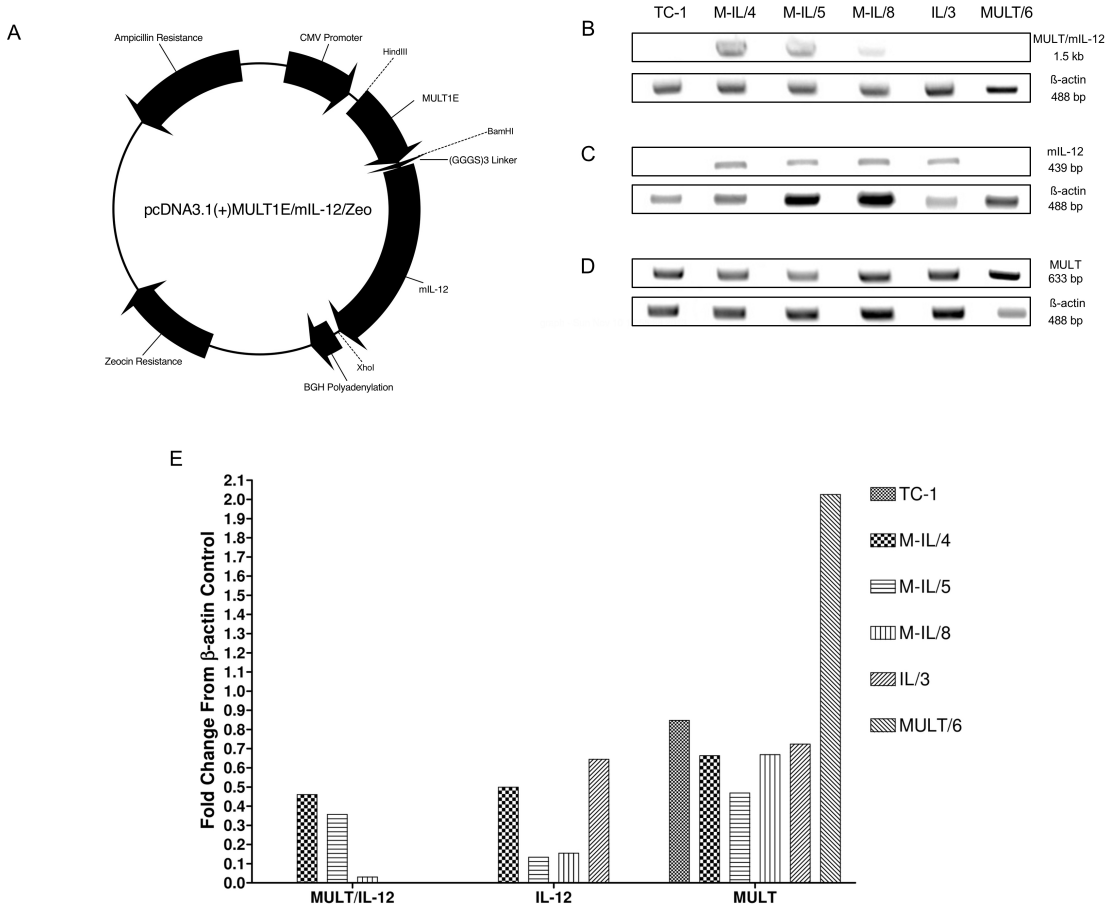

Figure 3.1. Plasmid pcDNA3.1(+)MULT1E/mIL-12/Zeo. Mouse interleukin 12 (mIL-12) was cloned from pORF-mIL-12 and a (GGGS)3 linker was added using PCR. mIL-12 was then inserted into the plasmid pcDNA3.1(+)MULT1E/Neo to form the plasmid pcDNA3.1(+)MULT1E/mIL-12/Neo. The fusion gene MULT1E/mIL-12 was then cut from the plasmid and inserted into pcDNA3.1(+)Zeo to form pcDNA3.1(+)MULT1E/mIL-12/Zeo (A). RT-PCR analysis of clones with transgenes. Total RNAs were isolated from each clone using primers to amplify a 1.6kb portion of the MULT1E/mIL-12 fusion gene containing 57 bp of MULT1E, the (GGGS)3 linker, and 1545 bp of mIL-12 (B), a 439 bp sequence near the 3' end of mIL-12 (C), or the entire 633 bp MULT1E sequence (D). Densitometry measurements of the bands for the three gene expression was performed and presented (E). All reactions included the β-actin housekeeping gene as controls (488 bp).

transcript than M-IL/8 (Figures 3.1b and e). It cannot be determined with RT-PCR if there is a difference between the background MULT1E transcription level found in the TC-1 control cells and the MULT1E transcription levels of the clones. To determine this, quantitative RT-PCR (qRT-PCR) was employed. Qiagen's QuantiTect SYBR Green RT-PCR kit was used to find relative quantification of' MULT1E mRNA transcripts using the housekeeping gene β-actin. The manufacturer's directions were followed using 500 ng total RNA and primers to amplify an 80 bp sequence at the 3′ end of MULT1E. The M-IL clones each show a significant increase in MULT1E mRNA transcription with M-IL/4 having the highest level and M-IL/8 having the lowest level (Table 3.1). This is consistent with the RT-PCR results above for the MULT1E/mIL-12 fusion gene. The IL/3 clone, however, also has a statistically significant increase in MULT1E mRNA transcription. To further confirm the transgene expression, cells of these clones were fixed, permeabilized and stained with both PE rat anti-mIL-12 and the chimeric protein mNKG2D/hIgG1 followed by fluorescein isothiocyanate antimouse NKG2D. In consistence with the PCR results, clone M-IL/4 expressed the highest levels of the fusion protein and M-IL/5 expressed intermediate levels of the fusion protein, whereas the fusion protein expression by clone M-IL/8 was not detectable (Figure 3.2). As expected, clone IL/3 only expressed mIL-12 and clone MULT/6 only expressed MULT1E (Figure 3.2)

The secretion of the transgene products was detected with a sandwich ELISA with anti-mouse MULT1 as the capturing antibody and anti-mIL-12 conjugated to PE as the detecting antibody. Consistent with RT-PCR data and fluorescent microscopy, clones

Table 3.1. qRT-PCR analysis of clones for MULT-1 gene expression. Qiagen's QuantiTect SYBR Green RT-PCR kit was used to find relative quantification of MULT1E mRNA transcripts using the housekeeping gene B-actin as a control. Primers were used to amplify an 80bp sequence at the 3' end of MULT1E. The data was analyzed using the comparative C_T method using the formula $2^{-\Delta\Delta C_T}$.[99]

Clone	Fold difference from TC-1 control	p-value
M-IL/4	20	$p<0.001$
M-IL/5	8	$p<0.01$
M-IL/8	4	$p<0.05$
IL/3	16	$p<0.001$

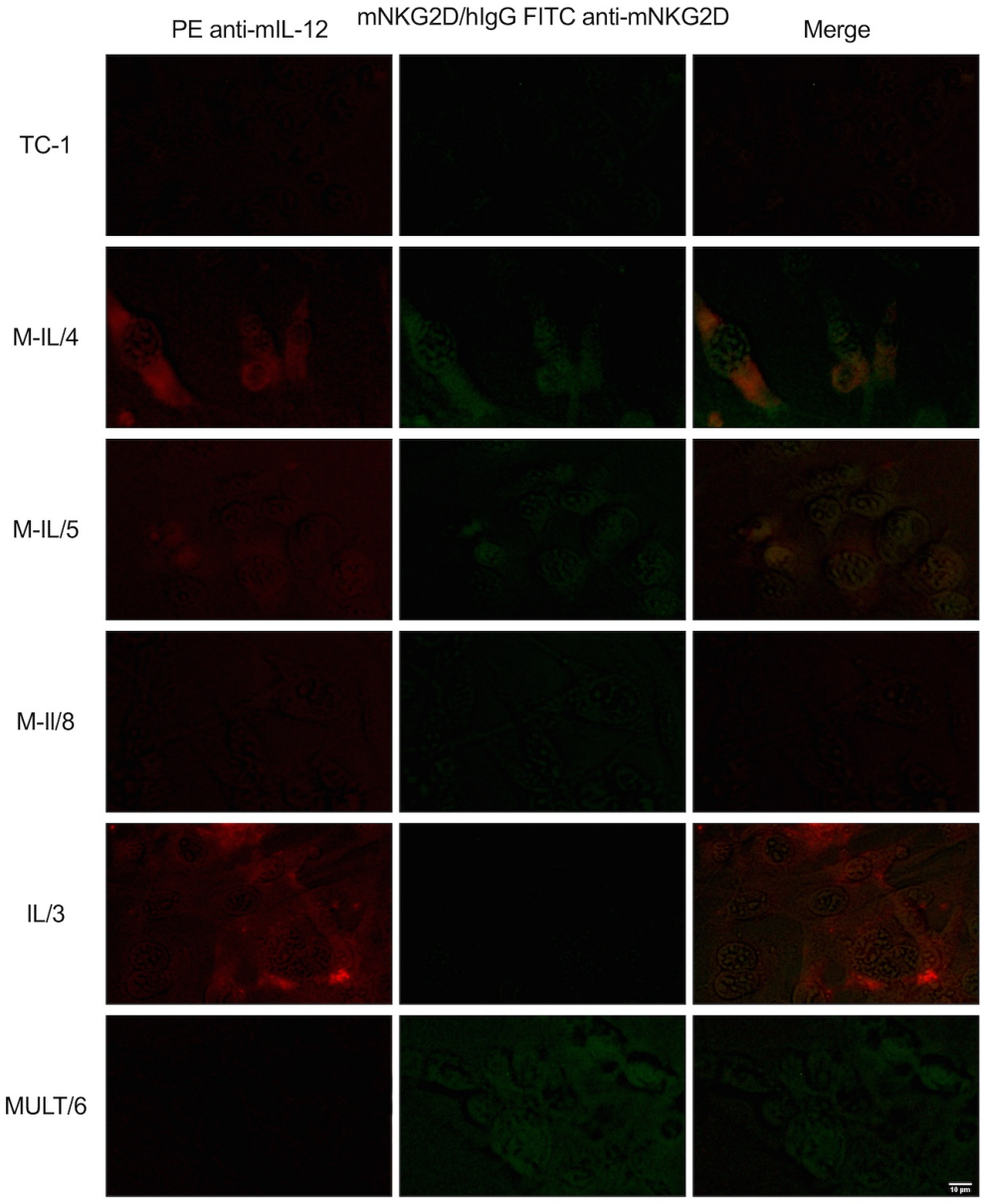

Figure 3.2. **Fluorescent microscopy analysis of clones with transgenes. To detect the production of the transgene products by the selected clones, cells were fixed, permeabilized, and stained with rat anti-mouse IL-12 (p40/p70) antibody conjugated with PE and the chimeric protein mNKG2D/hIgG$_1$ for 1 hour at room temperature followed by staining with anti-mouse NKG2D conjugated with FITC.**

M-IL/4 and M-IL/5 produced significant levels of the MULT1E/ mIL-12 fusion protein and successfully released it into the supernatant with M-IL/4 producing more than M-IL/5. Clones M-IL/8, IL/3 and MULT/6 did not produce detectable amounts of the fusion protein (Figure 3.3a). ELISA was also performed to detect mIL-12 or MULT1E separately. Clones M-IL/4 and IL/3 both produced similar amounts of mIL-12 and, therefore, IL/3 was used as the mIL-12 control clone for later studies. Compared with clone M-IL/4, clone M-IL/5 produced lower levels of mIL-12. In concurrence with the fluorescent microscopy data and the MULT1E/mIL-12 ELISA, clones M-IL/8 and MULT/6 did not produce detectable levels of mIL-12 (Figure 3.3b). The secreted form of MULT1E was detected at significant levels in clones M-IL/4, M-IL/5 and MULT/6 (Figure 3.3c). MULT/6 produces similar amounts of MULT1E as M-IL/5 and will be used as the MULT1 control.

In vitro biological activity of the MULT1E/mIL-12 fusion protein

Mouse NK cells were purified using negative selection from spleen cells and cocultured with clones expressing the fusion protein, mIL-12 or MULT1E and an IFN-γ ELISA was used to detect NK cell activation (Figure 3.3d). Clones M-IL/4, M-IL/5 and IL/3 all activated NK cells to produce significantly higher levels of IFN-γ than the control TC-1 cells. Clone M-IL/5 stimulated production of significantly more IFN-γ than clones M-IL/4 and IL/3 ($P<0.001$). Clone M-IL/4 stimulated production of similar levels of IFN-γ as clone IL/3. Neither clone M-IL/8 nor clone MULT/6 stimulated significant production of IFN-γ by NK cells. In order to determine whether the activated NK cells

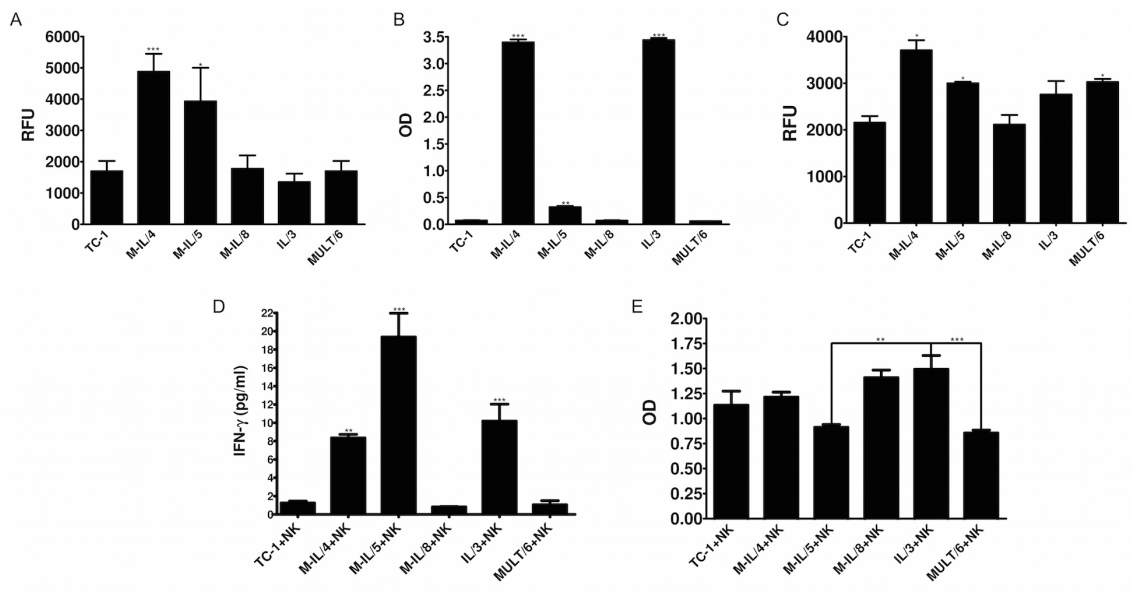

Figure 3.3. **Detection of secreted transgene products. Supernatant from each clone was collected for ELISA analysis. The fusion gene was detected using an antibody to MULT1 as the capturing antibody and anti-mIL-12 antibody conjugated with PE as the detecting antibody (A). mIL-12 was detected using an mIL-12 p70 Ready-SET-Go! ELISA kit following the manufacturer's directions (B). MULT1 was detected using an antibody to MULT1 as the capturing antibody and rmNKG2D was used as the detecting reagent followed by an anti-mNKG2D antibody conjugated with FITC (C). Clones were compared using a two-way ANOVA with Tukey's post-test. NK cell activation. Fresh mNK cells were cocultures with different clones in a 96 well plate at a ratio of 3:1 and incubated for 48 hours. After the incubation, media was removed and analyzed for the presence of IFN-γ using ELISA (D). The NK cells' cytotoxicity was determined by measuring the proliferation of the tumor cells using Promega's CellTiter 96 AQ_{ueous} nonRadioactive cell proliferation assay following the manufacturer's instructions (E). Clones were compared using a one-way ANOVA with Tukey's post-test for panel A and student t test for panel B. *p<0.05, **p<0.01, and ***p<0.001.**

can kill tumor cells expressing different transgene products, tumor cell proliferation was also measured using the MTS assay. NK cells killed significantly more tumor cells of clone M-IL/5 and clone MULT/6 (Figure 3.3e).

In vivo antitumor activity of tumor cells producing MULT1E/mIL-12 fusion protein

In order to determine whether the fusion protein can inhibit tumor growth *in vivo*, tumor cells expressing the fusion proteins, mIL-12 or MULT1E were intravenously injected into female C57BL/6J mice, respectively, and after 4 weeks, the mice were euthanized and their lungs were excised (Figure 3.4a). Lung volume was measured and the number of surface tumor nodules was counted. When compared with the control TC-1 cells, all the clones generated significantly fewer tumor nodules (Figure 3.4b). Among the clones, M- IL/4 did not grow any lung surface nodules, whereas clone M-IL/5 grew significantly fewer nodules than clone M-IL/8 or clone IL/3. Lung weight was also measured to include any tumors growing within the lungs (Figure 3.4c). The total lung weights of clones M-IL/4 and M-IL/5 are significantly lighter than clone M-IL/8.

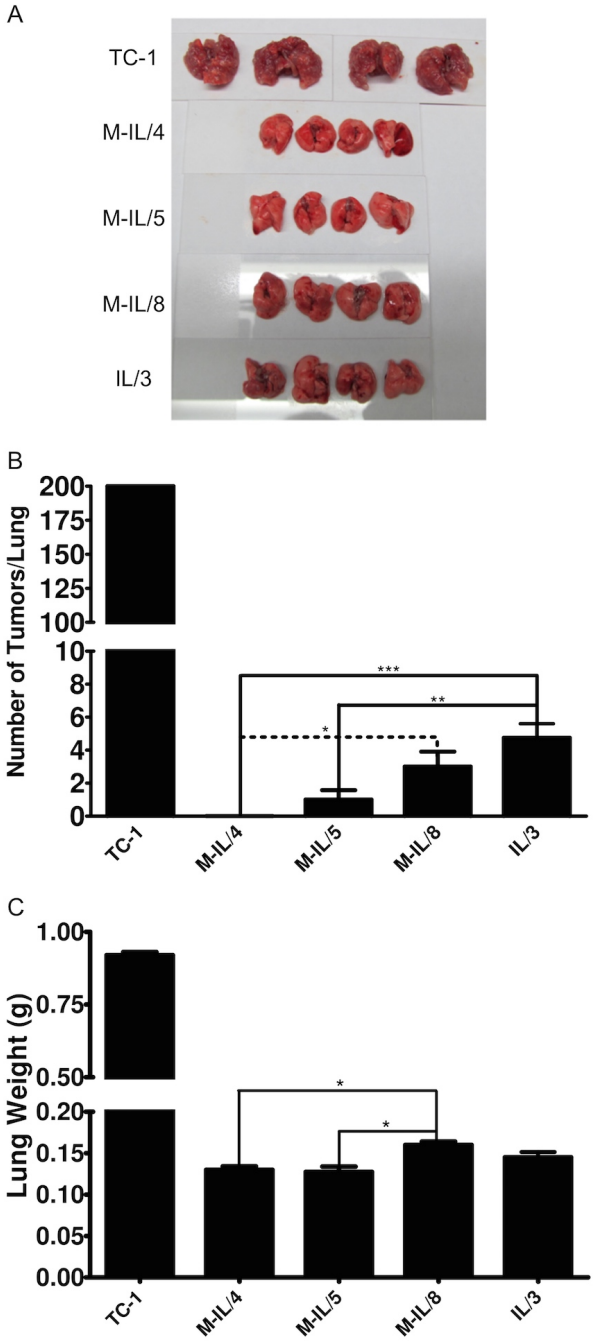

Figure 3.4. ***In vivo*** **antitumor activity of MULT1E/mIL-12 fusion protein. C57BL/6 female mice were injected intravenously with 1-2x10^5 cells in 0.5ml HBSS from each clone with TC-1 as a control. Four weeks later, the mice were euthanized and the lungs were excised (A) and fixed with 4% paraformaldehyde. The tumor nodules on each lung were counted using a dissecting microscope (B) and the tumor weight was recorded (C). Clones were compared using a one-way ANOVA with Tukey's post-test. *p<0.05, **p<0.01, and ***p<0.001.**

Discussion

Because NK cells have an important role in tumor immunosurveilance, many strategies have been employed to harness their ability to prevent neoplastic growth. Most of these strategies failed to provide significant clinical benefit or the toxicity experienced by patients outweighed any benefit.[96] In this study, we developed a bifunctional fusion protein, MULT1E/mIL-12, and hypothesized that it would effectively activate NK cells and other NKG2D-expressing killer cells in the tumor environment.

Screening for intracellular protein by fluorescent microscopy indicates that clones M-IL/4, M-IL/5 and M-IL/8 express varied levels of the fusion protein with clone M-IL/4 producing the most and clone M-IL/8 producing the least (Figure 3.2). RT-PCR and qRT-PCR confirm that this difference originates at the transcription level (Figures 3.1b–e and Table 3.1). There is also a background level of endogenous MULT1 transcription found in all clones. This background MULT1 transcription level is significantly increased in clone IL/3 (Table 3.1). This could be a result of the stress of transfection. Previous studies have shown that MULT1 is regulated post-transcriptionally; therefore, an increase in mRNA expression might not necessarily correlate with an increase in protein expression.[43]

It is important to demonstrate that the fusion protein is being released from the cell so it can interact with NK cells in the tumor microenvironment. To test this, supernatant was collected and screened using ELISA. The protein secretion data confirm that the protein being produced within the cell is also being released from the cell into the

supernatant (Figures 3.3a–c). A significant difference in the fusion protein expressed by clone M-IL/5 was observed when using the two different ELISA assays (Figures 3.3a and b). We suspect that the difference is due to the capture antibody for MULT1 in Figure 3.3a is not sufficient to detect all the proteins in the supernatants, whereas the IL-12 ELISA in Figure 3.3b is able to catch more, if not all, of the fusion proteins in the supernatants. ELISA data also confirm that although there is an increase in MULT1E transcripts in clone IL/3, there is no significant increase in the secreted form of MULT1E (Figure 3.3c).

To determine whether the fusion protein MULT1E/mIL-12 has enhanced ability to activate NK cells *in vitro*, freshly isolated NK cells were cocultured with clones expressing the transgenes. The amount of IFN-γ produced by NK cells was evaluated with ELISA to determine the level of NK cell activation. Clones M-IL/4, M-IL/5 and IL/3 produced significantly higher levels of IFN-γ than the control TC-1 cells. It is interesting to note that although clone M-IL/5 expressed lower levels of the fusion protein than clone M-IL/4 (Figure 3.2 and Figures 3.3a–c), it stimulated production of significantly more IFN-γ than clones M-IL/4 and IL/3 (Figure 3.3d). Further studies are needed to understand why this happened. Clone M-IL/4 stimulated production of similar levels of IFN-γ as clone IL/3, which is expected because they both have similar mIL-12 expression levels as shown in the mIL-12 ELISA (Figure 3.3b). Neither clone M-IL/8 nor clone MULT/6 stimulated significant production of IFN-γ. This is expected because the mIL-12 pathway, not MULT, is responsible for initiating the production of IFN-γ.[67,68]

To determine whether the fusion protein has the ability to enhance NK cell target cell killing activity, Promega's CellTiter 96 AQ_{ueous} nonRadioactive cell proliferation assay was used to measure the amount of target cells remaining after incubating with NK cells. The proliferation of clone M-IL/5 showed a significant decrease when compared with the clone that only produces mIL-12 (IL/3; Figure 3.3e). The decrease seen in clone M-IL/5 is also similar to the decrease seen in the MULT1E control clone (MULT/6). This suggests that although the IL/3 clone is able to increase the production of IFN-γ by NK cells, the addition of the MULT1E increases the cytotoxicity. This indicates that the mIL-12 moiety is responsible for initiating IFN-γ production and the MULT1E is responsible for activating NK cell-mediated cytotoxicity. It is expected that the *in vivo* increase in IFN-γ production will be able to modify other cell types not present in the *in vitro* model to aid in tumor cell killing. This should amplify the activity of the MULT1E/mIL-12 fusion protein.

To determine whether MULT1E/mIL-12 can activate NK cells to kill more tumor cells *in vivo*, tumor cells expressing the fusion protein were intravenously injected into mice and tumor burden was compared with mice receiving the parental tumor cells. After 4 weeks, the mice were euthanized and their lungs were excised. Lung volume was measured and the number of surface nodules was counted. There was a significant decrease in tumor count seen with all clones when compared with the parental TC-1 cells (Figure 3.4a). Although tumor count among the clones is very low, there appears to be a dose-dependent relationship in the MULT1E/mIL-12 clones. The higher expression of MULT1E/mIL-12 of clone M-IL/4 also seems to be more effective than the mIL-12 alone

(clone IL/3; Figure 3.4b). High expression of MULT1E/IL-12 fusion protein (clones M-IL/4 and M-IL/5) also showed a decrease in total lung weight compared with low fusion protein expression (clone M-IL/8; Figure 3.4c). It is also interesting to note that although clone IL/3 generated more tumor nodules on lungs (Figure 3.4b) than clone M-IL/8, the average lung weight of clone IL/3 is slightly lower than clone M-IL/8 (Figure 3.4c). We speculate that clone IL/3 tumors are smaller than clone M-IL/8 tumors, suggesting a possibility that NK cells or other NKG2D-expressing cells are activated after the tumor cells metastasized to the lung.

An interesting phenomenon observed in this report is the inconsistency of *in vitro* expression, *in vitro* NK cell activation and *in vivo* tumor inhibition between clones M-IL/4 and M-IL/5. Clone M-IL/4 expressed higher levels of fusion protein (Figure 3.3a), whereas clone M-IL/5 stimulated more IFN-γ by NK cells and inhibited more tumor growth *in vitro* (Figures 3.3d and e). In contrast, clone M-IL/4 inhibited more tumor growth than clone M-IL/5 *in vivo*. Although more studies are needed to clarify this inconsistency, it can be speculated that the NK cell activation and its anti-tumor mechanisms are different between *in vitro* and *in vivo*.

Two questions remain to be answered. First, are NK cells activated by the fusion protein *in vivo*? To answer this question, cells from different clones will be inoculated in to NK cell-deficient beige KO mice (C57BL/6J-Lystobg-J/J)[97] and tumor development will be monitored. We speculate that the tumors will grow faster in beige mice when compared with regular C57 BL/6J mice. Second, these pulmonary metastasis data cannot

differentiate whether the fusion protein prevents implantation of the tumor cells by causing NK cells to destroy them en route or if NK cells destroy the tumor cells post-implantation. To answer this question, we plan to develop a lentiviral vector or an adenoviral vector to deliver this fusion gene directly to established tumors. This will also provide pre-clinical data as lentiviral vectors and adenoviral vectors are already approved for clinical trials.[98] We will also investigate the possibility of using nanoparticles to deliver the fusion gene specifically to tumors. When the efficacy of the fusion gene and its delivery method is confirmed in animal models, this therapy could easily be converted for use in human immunotherapy by replacing MULT1E with major histocompatibility complex class I-related molecule A, a human NKG2D ligand, and mIL-12 with hIL-12. Another concern is the side effects of using this bifunctional protein. Although we did not observe any side effects in the current study, it should be a focus in the future *in vivo* studies, especially when viral vectors or nanoparticles are used to deliver the fusion gene into tumors. One of the key concerns will be how to make sure the gene is delivered into tumor cells specifically, but not into other healthy cells because if the gene is expressed by healthy cells, the fusion protein may activate NK cells in healthy tissues and cause off target effects.

In our previous studies, we created a fusion gene consisting of the extracellular domain of MULT1 and the transmembrane and introcellular domains of mouse Fas and demonstrated that fusion protein MULT1E/mFasTI activates NK cells and induces apoptosis of tumor cells expressing the protein *in vitro* and *in vivo*.[59] The limitation of fusion protein MULT1E/mFasTI is that it can only activate the NK cells that are engaged

with the tumor cells expressing the protein and it cannot activate other NK cells in the tumor environment. The bifunctional protein created in this study, however, can provide a local high dose of IL-12, which is able to activate NK cells in the local tumor environment so that the therapy may be more effective.

In conclusion, a fusion protein containing the extracellular domain of the NKG2D ligand MULT1 and mIL-12 was successfully created and can be stably expressed by TC-1 tumor cells. The fusion protein can increase the production of IFN-γ by NK cells *in vitro* and also increase the cytotoxicity when compared with clones expressing only mIL-12. Preliminary studies show that the anti-tumor activity of the MULT1E/mIL-12 fusion protein was more effective *in vivo* than clones expressing mIL-12 alone when the cells were introduced intravenously. The MULT1E/mIL-12 fusion protein may be a novel and effective anti-tumor therapy.

Materials and Methods

Cells and animals

The mouse lung carcinoma cell line TC-1 (ATCC no. CRL-2785) was cultured in RPMI 1640 medium containing 10% fetal bovine serum and 100 μg/ml gentamicin at 37^{o}C with 5% CO_2. Freshly isolated NK cells were maintained in RPMI 1640 containing 10%FBS, 50 μM β-mercaptoethanol, 2 mM glutamine, and 10 ml/L 100 x nonessential amino acids at 37^{o}C with 5% CO_2.

C57BL/6J female mice were housed in a pathogen-free animal facility. The animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication number 85–23) and the institutional guidelines.

Construction of pcDNA3.1(+)MULT1E/mIL-12/Neo Vector

The mIL-12 sequence was amplified from pORF-mIL-12 (InvivoGen, San Diego, CA) using the 5' primer CGCGGATCCGGTGGTGGTTCTGGTGGTGGTTCTGGTGG TGGTTCTAGGGTCATTCCAGTCTCTGGACCTGCCAGGTGTCTTAGCC and the 3' primer CCTCGAGCTAGGATCGGACCCTGCAGGG. The 5' primer contains a restriction cut site for BamHI and a (GGGS)3 linker to separate the two functional domains of the fusion protein. The 3' primer contains the restriction cut site for XhoI. The signal sequence and start codon were not amplified. PCR was performed to amplify mIL-12 using Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA). The fragment was excised and gel purified using a gel purification kit

(Qiagen, Valencia, CA, USA). Double enzyme digestion was performed on the purified fragment using BamHI and XhoI. The plasmid pcDNA3.1(+)MULT1E/Neo, created earlier in our lab,[59] was digested with BamHI and XhoI allowing for the insertion of mIL-12 directly downstream of MULT1E in frame. The linearized plasmid was excised and gel purified using a gel purification kit (Qiagen, Valencia, CA, USA). The enzyme digested mIL-12 PCR fragment was then ligated to the pcDNA3.1(+)MULT1E/Neo creating the new plasmid pcDNA3.1(+)MULT1E/mIL-12/Neo (Figure 3.1A). The sequence was confirmed using DNA sequencing.

Construction of pcDNA3.1(+) MULT1E/mIL-12/Zeo Vector

To construct the zeocin resistant vector necessary to transfect TC-1 cells, pcDNA3.1(+)MULT1E/mIL-12/Neo and pcDNA3.1(+)Zeo (Invitrogen, Carlsbad, CA, USA) were digested with HindIII and XhoI. The linear vector of pcDNA3.1(+)Zeo and fragments containing MULT1E/mIL-12 from pcDNA3.1(+) MULT1E/mIL-12/Neo were then excised and gel purified using a gel purification kit (Qiagen, Valencia, CA, USA). They were then ligated creating the plasmid pcDNA3.1(+)MULT1E/mIL-12/Zeo

Construction of pcDNA3.1(+)mIL-12/Zeo Vector

To make a control mIL-12 vector, the mIL-12 sequence was again amplified from pORF-mIL-12 (InvivoGen, San Diego, CA). However, the start codon and signal sequence need to be reserved, so a different 5' primer (CCAAGCTTCCATGGGTCAAT CACGCTACCTCC) with a HindIII cut site at the 5' end was used. The original 3' primer with the restriction cut site for XhoI was also used. PCR was performed to

amplify mIL-12 using Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA). The fragment was excised and gel purified using a gel purification kit (Qiagen, Valencia, CA, USA). Double enzyme digestion was performed on the purified fragment using HindIII and XhoI.

pcDNA3.1(+)Zeo was digested with HindIII and XhoI allowing for the insertion of mIL-12. The fragment was excised and gel purified using a gel purification kit (Qiagen, Valencia, CA, USA). The mIL-12 sequence was then ligated to pcDNA3.1(+)Zeo (Invitrogen, Carlsbad, CA, USA) creating the new plasmid pcDNA3.1(+)mIL-12/Zeo. The sequence was confirmed using DNA sequencing.

Construction of pcDNA3.1(+)MULT1ES/Zeo

To make a control MULT1E vector encoding secretable MULT1E, the MULT1E sequence was amplified from pcDNA3.1(+)MULT1E/Neo. The 5' primer (CCCAAGCT TATGGAGCTGACTGCCAGTAACAAGGTCC) included a restriction site for HindIII and the 3' primer (CCCTCGAGCTAGGTACTGAA) included a stop codon and the restriction site for XhoI. PCR was performed to amplify MULT1E using Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA). The fragment was excised and gel purified using a gel purification kit (Qiagen, Valencia, CA, USA). Double enzyme digestion was performed on the purified fragment using HindIII and XhoI.

pcDNA3.1(+)Zeo (Invitrogen, Carlsbad, CA, USA) was digested with HindIII and XhoI allowing for the insertion of MULT1E. The fragment was excised and gel purified

using a gel purification kit (Qiagen, Valencia, CA, USA). The MULT1E sequence was then ligated to the pcDNA3.1(+)Zeo creating the new plasmid pcDNA3.1(+)MULT1E/Zeo. The sequence was confirmed using DNA sequencing.

Cell transfection

TC-1 cells were transfected with either pcDNA3.1(+)MULT1E/mIL-12/Zeo, pcDNA3.1(+)mIL-12/Zeo, or pcDNA3.1(+)MULT1E/Zeo using Lipofectamine (Invitrogen, Carlsbad, CA, USA) as directed by the manufacturer. To obtain stable clones, the transfected TC-1 cells were cultured in medium containing 200 μg/ml zeocin. Drug-resistant clones were collected and subcultured in the presence of the appropriate drug.

Proliferation Assay

To determine if the selected clones grow at the same rate as the untransfected cells, cells were counted every day for 5 days. On day one, $0.04x10^6$ cells were plated on 12 well plates in triplicates. At each time point, 3 wells of each clone were trypsinized and counted using a hemocytometer. This test was preformed twice to obtain 6 replicates. Clones were compared using a two-way ANOVA with Tukey's post-test.

RT-PCR

Total RNA was extracted from each clone using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's directions. cDNA was produced from 2ug of the total RNA using the QuantiTect RT (QIAgen, Valencia, CA, USA) following

the manufacturer's directions. Four microliters of the product was then added to a PCR reaction containing primers to amplify a 1.6kb portion of the MULT1E/mIL-12 fusion gene containing 57 bp of MULT1E, the (GGGS)3 linker, and 1545 bp of mIL-12. A Phusion High-Fidelity DNA Polymerase kit (New England BioLabs, Ipswich, MA, USA) was used with the 5' primer GATGAGCTCATGTTGCACTG and the 3' primer CATTC CCGCCTTTGCATTGGAC. Similar reactions were carried out to amplify a 439 bp sequence near the 3' end of mIL-12 (using the 5' primer GCAGTGACATGTGGAATG GC and the 3' primer CATTCCCGCCTTTGCATTGGAC) and the entire 633 bp MULT1E sequence (using the 5' primer CCCAAGCTTATGGAGCTGACTGCCAGTA ACAAGGTCC and the 3' primer CCCTCGAGCTAGGTACTGAA). All reactions included the β-actin housekeeping gene. The density of the DNA bands were quantified using the built-in software from the Fotodyne gel doc system, FOTO/Analyst FX.

qRT-PCR

Qiagen's QuantiTect SYBR Green RT-PCR kit was used to find relative quantification of MULT1E mRNA transcripts using the housekeeping gene β-actin. The manufacturer's directions were followed using 500ng total RNA, the 5' primer GACTGC AGGACCTGGCTTGATGAGC, and the 3' primer CCCTCGAGCTAGGTACTGAA to amplify an 80bp sequence at the 3' end of MULT1E. The reaction was carried out in a Mastercycler Ep Realplex (Eppendorf, Hamburg, Germany) and analyzed using the comparative C_T method using the formula $2^{-\Delta\Delta C_T}$.[99]

Fluorescent Microscopy

To detect the production of the transgene products by the selected clones, $3x10^4$ cells of each clone were plated in 96 well plates and incubated at 37°C overnight. After the incubation time, the media was removed and the cells were washed twice with staining buffer (PBS, 0.1%BSA, and 0.1% sodium azide). Cells were fixed by incubating cells for 30 min at 4°C with fixation buffer (BD Pharmingen, San Jose, CA, USA). The fixation buffer was then removed and the cells were washed with pre-warmed permeabilization buffer (BD Pharmingen, San Jose, CA, USA). The cells were then incubated with permeabilization buffer for 30 min at 37°C. After washing twice with staining buffer, the cells were stained with PE rat anti-mouse IL-12 (p40/p70) (2.5μg/ml) (BD Pharmingen, San Jose, CA, USA) and the chimeric protein mNKG2D/hIgG$_1$ (1μg/ml) (R&D Systems, Minneapolis, MN, USA) for 1 hour at room temperature. The cells were then washed three times with staining buffer. FITC anti-mouse NKG2D (2.5 μg/ml) (BioLegend, San Diego, CA, USA) was then added and incubated for an hour in the dark. The cells were then washed five times with staining buffer and observed with an Olympus 1x70 fluorescent microscope.

Detection of Secreted Protein

Cells of different clones were plated at $2x10^6$ cells in 6 well plates with normal growth media on day one. On day two, the media was removed, the cells were rinsed with PBS, and serum free RPMI was added. On day three, the serum free RPMI was removed and centrifuged at 1000g for 15 min. The supernatant was then tested using an

appropriate immunofluorescence or ELISA protocol.

To detect the presence of MULT1E/mIL-12, 96 well plates were coated with 2 μg/ml mouse MULT1 mAb (Clone 237104) Rat IgG_2A (R&D Systems, Minneapolis, MN, USA) in carbonate coating buffer and incubated overnight at 4°C. The plates were rinsed with PBS and blocked with 10% FBS for 1.5 hours. After washing three times with PBS, 100μl of the cell supernatant was added to the plate in triplicates. The samples were incubated at room temperature for 3 hours. After removing the samples and washing the plates three times with PBS, 1 μg/ml PE rat anti-mouse IL-12 (p40/p70) (BD Pharmingen, San Jose, CA, USA) in blocking solution was added to the plates. The plates were then incubated for 1.5 hours at room temperature. After washing five times with PBS, the plates were read with a SpectraMax Gemini XS using SOFTMax Pro 3.1.2 software (Molecular Devices, Sunnyvale, CA, USA). Clones were compared using a one-way ANOVA with Tukey's post-test.

To detect the presence of MULT1E, 96 well plates were coated with 2 μg/ml mouse MULT1 mAb (Clone 237104) Rat IgG_2A (R&D Systems, Minneapolis, MN, USA) in carbonate coating buffer and incubated overnight at 4°C. The plates were rinsed with PBS and blocked with 10% FBS for 1.5 hours. After washing three times with PBS, 100μl of the cell supernatant was added to the plate in triplicates. The samples were incubated at room temperature for 3 hours. After removing the samples and washing the plates three times with PBS, 1 μg/ml mNKG2D/$hIgG_1$ chimeric protein (BD Pharmingen, San Jose, CA, USA) in blocking solution was added to the plates. The plates were then

incubated for 1.5 hours at room temperature. After washing five times with PBS, 1ug/ml FITC mouse anti-hIgG (BD Pharmingen, San Jose, CA, USA) in blocking solution was added to the plates and incubated for 1.5 hours at room temperature. After washing five more times with PBS, plates were read with a SpectraMax Gemini XS using SOFTMax Pro 3.1.2 software (Molecular Devices, Sunnyvale, CA, USA). Clones were compared using a one-way ANOVA with Tukey's post-test.

To detect the presence of secreted mIL-12 from the cells, a mIL-12 p70 Ready-SET-Go! ELISA kit was used following the manufacturer's directions (eBioscience, San Diego, CA, USA). Plates were read with a Bio-Rad model 680 microplate reader (Hercules, CA, USA). Clones were compared using a one-way ANOVA with Tukey's post-test.

Isolation of mouse NK cells

Spleens were collected from C57BL/6 mice. A single cell suspension was prepared using mechanical disruption with a 70 μm filter and centrifugation. All other cells were depleted by magnetic labeling using a MACS NK cell isolation kit (Miltenyl Biotec, Auburn, CA, USA).

IFN-γ production and Cytotoxicity of NK cells

Different clones and isolated mNK cells were plated on a 96 well plate at a ratio of 1:3 in 200 μl NK media and incubated for 48 hours. After the incubation, 50 μl of media was removed and analyzed for the presence of IFN-γ using BD OptiEIA mouse

IFN-γ ELISA kit II (BD Pharmingen, San Jose, CA, USA) following the manufacturer's directions. The rest of the media was removed from the cells and 100 µl fresh NK media was added. Cell proliferation was determined using Promega's CellTiter 96 AQ_{ueous} nonRadioactive cell proliferation assay (Madison, WI, USA) following the manufacturer's instructions. Clones were compared using a one-way ANOVA with Tukey's post-test.

In vivo antitumor activity of MULT1E/mIL-12 fusion protein

C57BL/6 female mice were injected intravenously with 1-2x10^5 tumor cells in 0.5ml HBSS. Four weeks later, the mice were euthanized and the lungs were excised and fixed with 4% paraformaldehyde. The tumor nodules on each lung were counted using a dissecting microscope and the tumor weight was recorded. Clones were compared using a one-way ANOVA with Tukey's post-test.

Statistics

GraphPad software (Prism, San Diego, CA, USA) was used to plot graphs. One-way or two-way analysis of variance (ANOVA) with Tukey's post-tests or student t test was used to perform the statistical analyses of the data. The significance was represented as $^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$.

Acknowledgements

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CHAPTER FOUR

MICA/IL-12: A NOVEL BIFUNCTIONAL PROTEIN FOR KILLER CELL ACTIVATION

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Abstract

Natural killer (NK) cells have the potential to be effective killers of tumor cells. They are governed by inhibitory and activating receptors like NKG2D, whose ligands are normally upregulated in cells that are stressed, like cancer cells. Advanced cancer cells, however, have ways to reduce these ligands' expression, leaving them less detectable by NK cells. Along with these receptors, NK cells also require activating cytokines, like IL-12. Previous work in our lab has shown that a fusion protein of the extracellular domain of mouse UL-16 binding protein-like transcript 1 (MULT1E) and mouse Interleukin 12 (IL-12) can effectively activate mouse NK cells in *in vitro* assays and in *in vivo* animal tumor models.[100] The goal of this study is to expand the concept of developing a novel bifunctional fusion protein for enhanced NK cell activation to human killer cells. The proposed protein combines the extracellular domain of a human NKG2D ligand, MHC class I polypeptide-related sequence A (MICA), and IL-12. It is hypothesized that when expressed by tumor cells, the protein will activate human NK and other killer cells using

the NKG2D receptor, and deliver IL-12 to the NK cells where it can interact with the IL-12R and enhance cytotoxicity. The fusion protein, when expressed by engineered tumor cells, indeed activated NK92 cells as measured by an increase in interferon-γ (IFN-γ) production and an increase in cytotoxicity of tumor cells. The fusion protein was also able to increase the proliferation of human peripheral blood mononuclear cells (PBMCs) and augment their production of IFN-γ. This study along with the data from the previous mouse studies suggests that the MICA/IL-12 bifunctional fusion protein represents an effective activator of killer cells for cancer treatment.

Introduction

Natural killer (NK) cells are part of the innate immune system and are one of the primary cell types involved in anticancer immunosurveillance. At the onset of neoplastic growth, tumor cells upregulate surface antigens, some of which are ligands for NK cell activating receptors, including NKG2D. Once NK cells are activated, cytotoxic granules are exocytosed into the extracellular space between the NK cell and the target cell and apoptosis of target cells is induced.[19] As the cancer progresses, however, advanced tumor cells have found ways to downregulate surface NKG2D ligands. It has been shown that the extracellular domains of MHC class-I related molecule A (MICA), a human NKG2D ligand, can be shed by tumor cells by proteolytic cleavages, thus preventing activation of cytotoxic immune cells.[52] The immune system may also aid in selecting against tumor cells that maintain NKG2D ligand expression.[5] Due to these properties, MICA serves as an excellent target for anti-cancer gene therapy.

Interleukin 12 (IL-12), also known as NK cell stimulatory factor 2, is an activator and initiator of NK cell proliferation. IL-12's activity is more efficient than either IL-2 or the IFNs, requiring picomolar instead of nanomolar concentrations.[65] In fact, NK cells genetically engineered to express IL-12 in a membrane anchored form by mouse sonic hedgehog C-terminal domain showed reduced (>10 fold) dependency on IL-2 and a significantly prolonged survival time for tumor bearing mice receiving an intravenous injection of these cells.[95] IL-12 has been shown consistently to have potent anti-tumor activity primarily through the activity of IFN-γ, the activation of $CD8^+$ T cells, and the

inhibition of angiogenesis.[67,68]

Previous work in our lab has shown that a bifunctional fusion protein containing the mouse NKG2D ligand mouse UL-16 binding protein-like transcript 1 (MULT1) and mouse IL-12 can effectively activate mouse NK cells both *in vitro* and *in vivo* leading to a reduction in tumor size. It is believed that the enhanced antitumor effect is due to the simultaneous activation of NK cells and other NKG2D expressing killer cells through the MULT1/NKG2D pathway and the IL-12/IL-12R pathway. In addition, MULT1 may also function as a carrier to deliver IL-12 directly to the killer cells. When the MULT1 domain binds to an NKG2D receptor on the surface of an NK cell, the IL-12 may also be held in close proximity to IL-12 receptors on the same cell, which will maintain a high level of IL-12 in the tumor microenvironment while providing low systemic IL-12 levels and reduced toxicity.[100]

For this work to be translated for clinical use, the protein needs to be adapted for use in humans. The purpose of this study is to develop a bifunctional fusion protein containing a human NKG2D ligand, MICA, and IL-12. Since mouse IL-12 is fully effective in engaging the human IL-12 receptor, a recombinant mouse IL-12 gene is used in this model study. [101] This human version of the fusion protein was first examined by transfecting the fusion gene into the human lung carcinoma cell line A549. Stable clones of tumor cells expressing the fusion protein and control IL-12 or MICA proteins were evaluated in a series of *in vitro* assays to determine their ability to activate the human natural killer cell line NK92 and isolated PBMCs.

Results

Construction of expression vectors

To construct the plasmid containing the MICA/IL-12 fusion gene, the MICA extracellular domain cDNA sequence was amplified from pCMV-SPORT6-MICA and used to replace MULT1E in the plasmid pcDNA3.1(+)MULT1E/mIL-12 that had been constructed earlier in our lab.[100] MICA was inserted directly upstream of a (GGGS)3 linker, allowing flexibility, and IL-12 in frame creating the new plasmid pcDNA3.1(+)MICA/IL-12 (Figure 4.1). The IL-12 control plasmid, pcDNA3.1(+)IL-12, was constructed by amplifying the IL-12 sequence from pORF-mIL-12 and ligating it into the pcDNA3.1(+) vector. Since the fusion protein is secretable and only contains the extracellular domain of MICA, PCR was used to amplify MICA from pcDNA3.1(+)MICA/IL-12 and add a stop codon at the end of the extracellular domain. Because MICA is a type I transmembrane protein and the signal sequence is conserved, the resulting protein should be transported outside of the cell.[102]

Transfection and stable clone selection

A549 cells were transfected with plasmids pcDNA3.1(+)MICA/IL-12 (Figure 4.1), pcDNA3.1(+)MICA, or pcDNA(+)IL-12, respectively, using Lipofectamine. Multiple clones from each transfection that were geneticin resistant were selected. RT-PCR and ELISA analysis (see below) were used to screen these clones and clones M-IL/4 and M-IL/19 were selected for fusion protein MICA/IL-12, clone IL/14 for IL-12, and

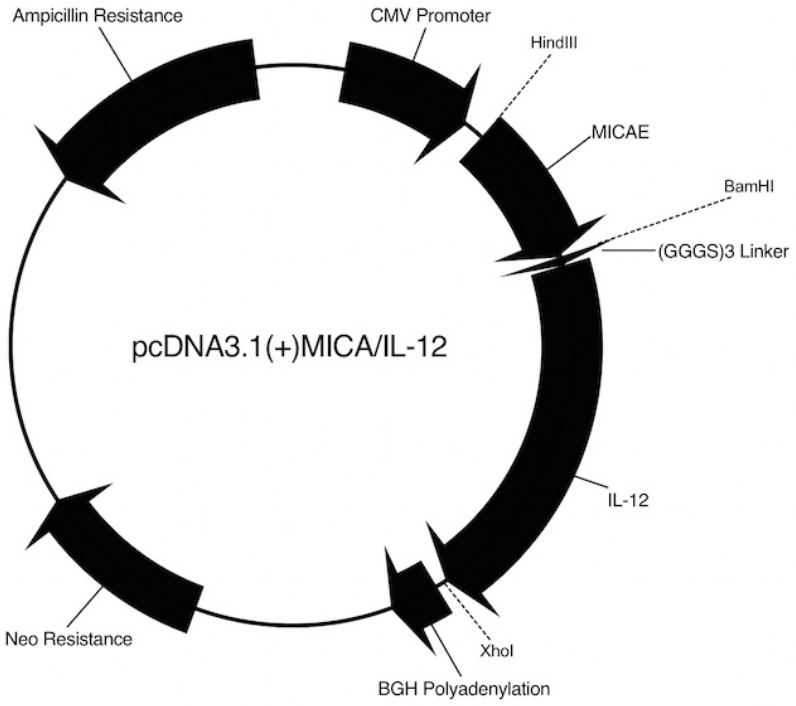

Figure 4.1. Plasmid pcDNA3.1(+)MICA/IL-12. The MICA extracellular domain sequence was amplified from pCMV-SPORT6-MICA and used to replace MULT1E in the plasmid pcDNA3.1(+)MULT1E/mIL-12 that had been created earlier in our lab.[100] MICA was inserted directly upstream of a (GGGS)3 linker and IL-12 in frame creating the new plasmid pcDNA3.1(+)MICA/IL-12.

clone M/19 for MICA. An *in vitro* cell proliferation assay shows that all clones grow at a similar rate (data not shown).

Stable Clones Express the Fusion Protein

The fusion protein expression was first examined by reverse transcription-PCR (RT-PCR) on total RNA samples collected from the clones using three pairs of primers. The first pair was specific to a 177 bp portion of the extracellular domain of MICA. The second pair amplified an 805 bp sequence of IL-12. The final primer pair amplified a 1.2 kb portion of the MICA/IL-12 fusion gene containing 446 bp of MICA, the (GGGS)3 linker, and 708 bp of IL-12. All cells, including the A549 control cells and IL/14, contain mRNA transcripts for MICA (Figure 4.2A). The fusion gene clones, along with the IL/14 clone contain mRNA transcripts for IL-12 (Figure 4.2B). Only clones M-IL/4 and M-IL/19 contain mRNA transcripts for the MICA/IL-12 fusion gene (Figure 4.2C).

A sandwich ELISA detected the secretion of the transgene products. The rhNKG2D/Fc chimera was used to coat the plate and anti-mouse IL-12 conjugated with PE was used as the detecting antibody. Clone M-IL/4 produced significant levels of the MICA/IL-12 fusion protein and successfully released it into the supernatant. Clone M-IL/19 did not produce detectable amounts of the fusion protein (Figure 4.3A). ELISA was also performed to detect IL-12 or MICA separately. Clones M-IL/4 and IL/14 both produced similar amounts of IL-12 (Figure 4.3B). M-IL/19 produced no detectable levels of IL-12. The secreted form of MICA was detected at significant levels in clones M-IL/4 and M/19 (Figure 4.3C).

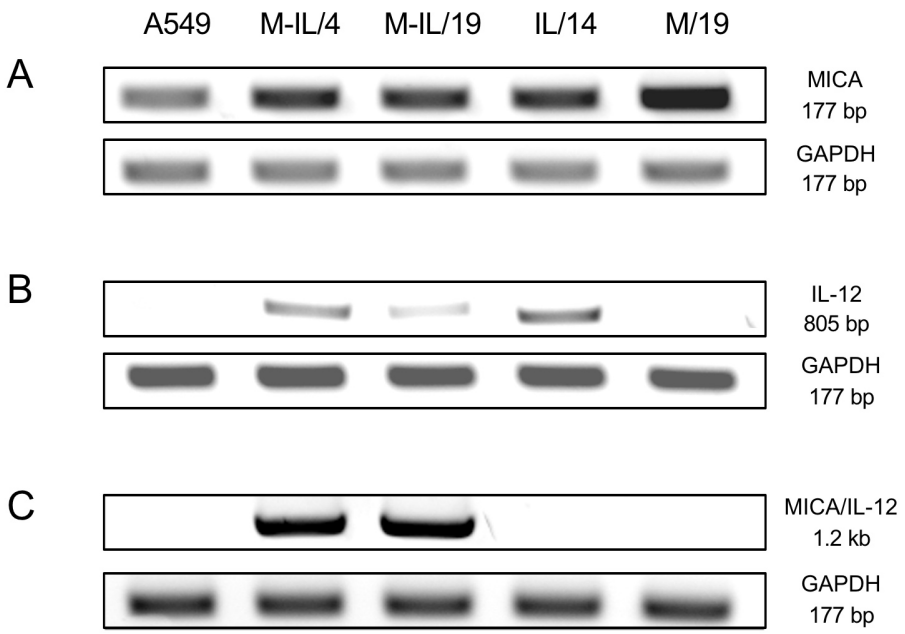

Figure 4.2 RT-PCR analysis of A549 clones with transgenes. Total RNAs were isolated from each clone and reverse transcription was used to create cDNA. Primers were used to amplify a 177 bp portion of the extracellular domain of MICA (A), a 805 bp portion of IL-12 (B), and a 1.2 kb portion of the MICA/IL-12 fusion gene containing 446 bp of MICA, the (GGGS)3 linker, and 708 bp of IL-12 (D) using PCR.

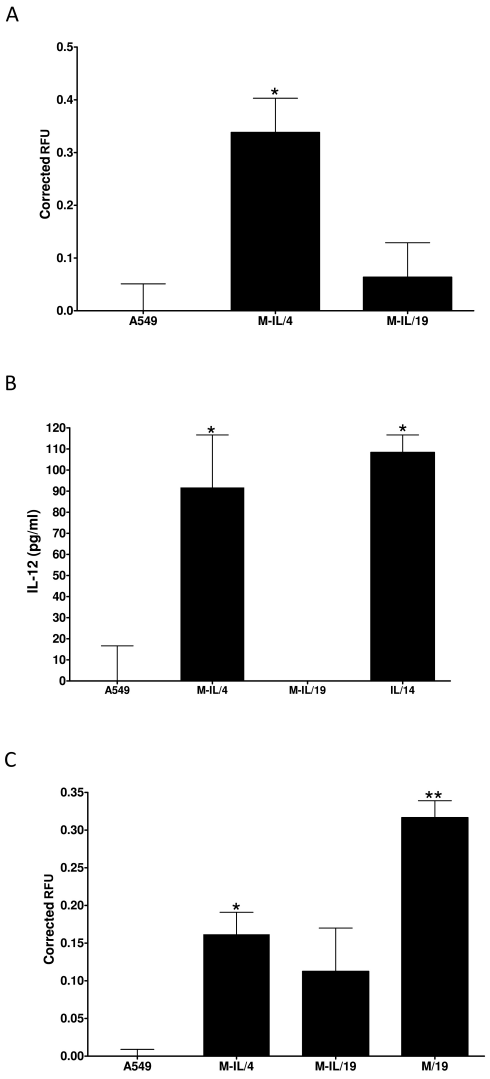

Figure 4.3. **Detection of secreted transgene products from A549 clones. Supernatant from each clone was collected for ELISA analysis. The fusion gene was detected by coating the plate with rhNKG2D/Fc chimera, adding the supernatant, and using anti-mIL-12 antibody conjugated with PE as the detecting antibody (A). IL-12 was detected using an mIL-12 ELISA kit (B). MICA was detected by coating the plate with rhNKG2D/Fc chimera, adding the supernatant, and using anti-hMICA phycoerythrin conjugated as the detecting antibody (C). Corrected RFU is calculated by subtracting background RFU from each value. Clones were compared using a one-way ANOVA with Tukey's post-test. *p<0.05 and **p<0.01.**

MICA/IL-12 activates NK92 cells

Human NK92 cells were co-cultured with the A549 cells and clones M-IL/4, M-IL/19, I/14, or M/19 for 48 hours. To determine if the proteins produced by these clones could activate the NK92 cells, two assays were performed. First, 100 μl of the media was removed and screened using an IFN-γ ELISA. The NK92 cells co-cultured with clones M-IL/4, IL/14, and M/19 showed significantly higher IFN-γ production than NK92 cells co-cultured with A549 parental cells. M-IL/19 was not able to significantly increase IFN-γ production (Figure 4.4A).

The second assay measured primed NK92 cells' cytotoxicity towards the tumor cell clones. After removing the supernatants from the co-cultures, the wells were gently rinsed five times with PBS to remove the remaining suspension NK92 cells and leave behind the adherent tumor cells. The remaining cells were then measured using an MTS cell proliferation assay. There were significantly fewer M-IL/4, M-IL/19, and IL/14 cells when compared to the A549 parental cells. There was no significant difference in the number of M/19 cells compared to the A549 parental cells (Figure 4.4B).

It is interesting to know if pre-activation or priming of NK92 cells with supernatant collected from the clones expressing the fusion protein, IL-12, or MICA can enhance the killing of parental A549 cells. A549 parental cells and the clones M-IL/4, M-IL/19, IL/14, or M/19 were cultured in NK media for 48 hours before collecting the supernatant. NK92 cells were then plated in the collected supernatants and allowed to

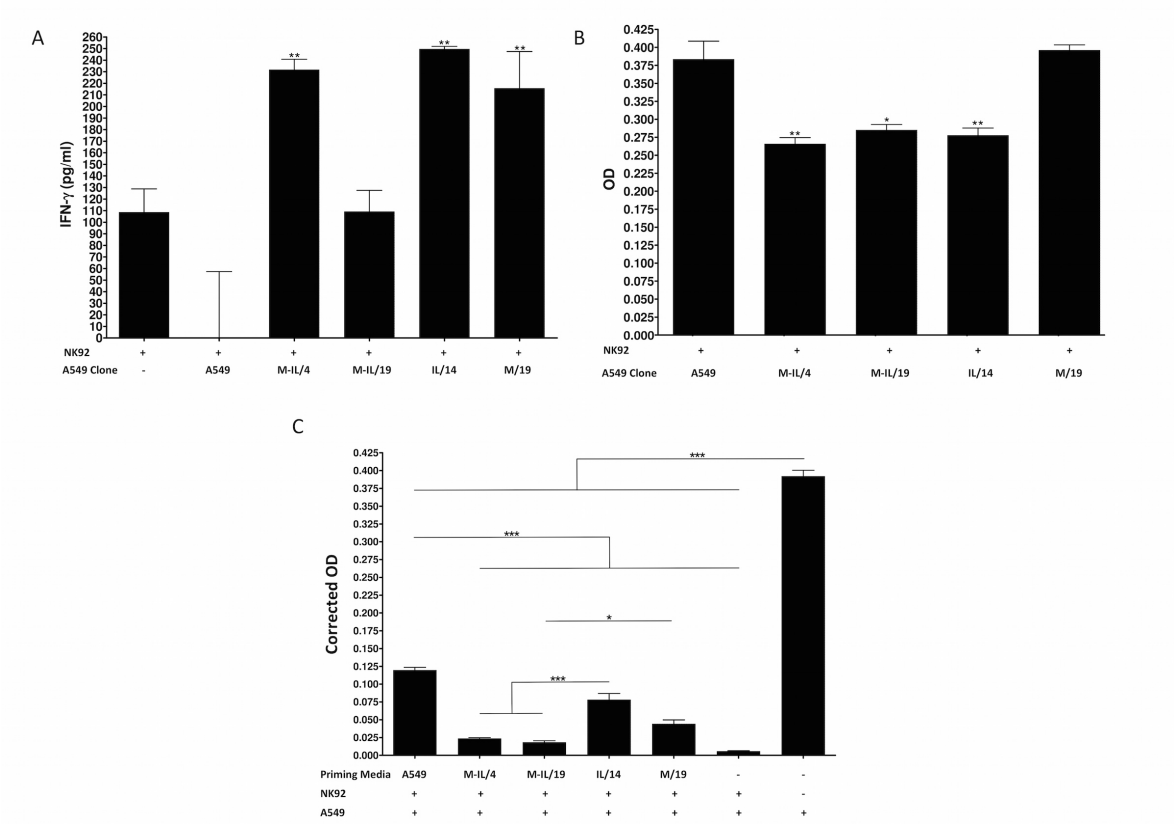

Figure 4.4. NK92 cell activation. NK92 cells were co-cultured with different clones in a 96 well plate at a ratio of 10:1 and incubated for 48 hours. After the incubation, media was removed and analyzed for the presence of IFN-γ using ELISA (A). The NK92 cells' cytotoxicity was determined by measuring the proliferation of the tumor cells using Promega's CellTiter 96 AQ_{ueous} nonRadioactive cell proliferation assay following the manufacturer's instructions (B). NK92 cells primed with media collected from A549 clones were co-cultured with parental A549 cells at a ratio of 10:1 for 24 hours. The amount of A549 cells remaining after the incubation time was again measured using Promega's CellTiter 96 AQ_{ueous} nonRadioactive cell proliferation assay following the manufacturer's instructions. Corrected OD is calculated by subtracting background OD from each value (C). Clones were compared using a one-way ANOVA with Tukey's post-test. *p<0.05, **p<0.01, and ***p<0.001.

incubate for 48 hours. These primed NK92 cells were then plated with parental A549 cells at a ratio of 10:1 and incubated for 24 hours. At the end of the incubation, the culture media was removed and the wells were gently rinsed five times to remove the suspension NK92 cells and leave behind the adherent A549 cells. The remaining cells were measured using an MTS cell proliferation assay. Although all NK92 cells, whether primed or not, were able to significantly reduce the tumor cell number compared to the control, M-IL/4 and M-IL/19 primed NK92 cells were able to kill significantly more tumor cells than either A549, IL-4, or M/19 primed NK92 cells. Interestingly, unprimed NK92 cells were the most effective killers of A549 cells (Figure 4.4C).

MICA/IL-12 activates human PBMCs

Human peripheral blood mononuclear cells (PBMCs) were isolated from leukapheresis product using the Ficoll-Paque method. These cells were then cultured with supernatants collected from A549 cells or clones M-IL/4, M-IL/19, M/19, or IL/14 for 10 days. To determine if the supernatants could promote PBMC proliferation, an MTS cell proliferation assay was performed. Only supernatant collected from clone M-IL/4 that expresses the fusion protein at a relatively high level was able to significantly increase PBMC cell number (Figure 4.5A). An IFN-γ ELISA was also used to detect killer cell activation. Clone M-IL/4 was also able to induce a significantly higher production of IFN-γ when compared to clones M-IL/19 and IL/14. Clone M/19 also induced production of a large amount of IFN-γ (Figure 4.5B). More interestingly, PBMCs without co-culture expressed the highest level of IFN-γ.

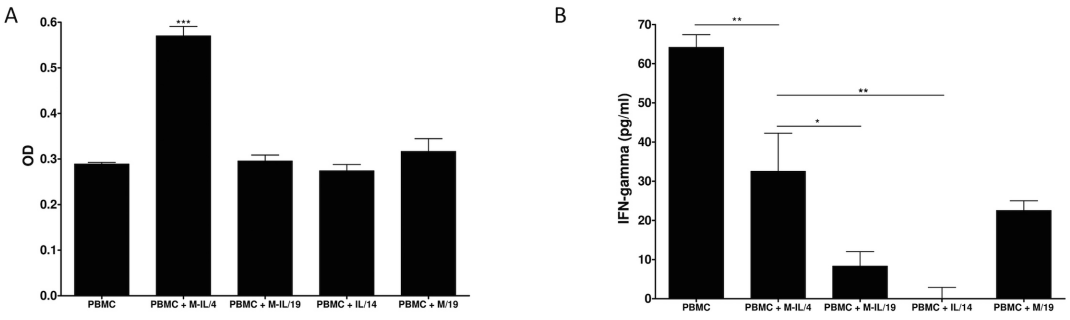

Figure 4.5. PBMC activation. Supernatant collected from A549 cells expressing the fusion protein, IL-12, or MICA was used to activate isolated human PBMCs. After the incubation the relative cell proliferation was determined using Promega's CellTiter 96 AQ_{ueous} nonRadioactive cell proliferation assay (A) and the media was removed and analyzed for the presence of IFN-γ (B). Clones were compared using a one-way ANOVA with Tukey's post-test. *p<0.05, **p<0.01, and ***p<0.001.

Discussion

Because NK cells play an important role in tumor immunosurveilance, many strategies have been employed to harness their ability to prevent neoplastic growth. In this study we developed a bifunctional fusion gene encoding MICA/IL-12, and hypothesized that the bifunctional protein, when expressed by tumor cells, would effectively activate NK cells and other NKG2D expressing killer cells in the tumor environment and increase cytotoxicity.

RT-PCR indicates that all clones contained mRNA for MICA (Figure 4.2A). This is not surprising since MICA is commonly expressed in lung carcinomas.[103] IL-12 mRNA is detected in the two fusion gene clones (M-IL/4 and M-IL/19) and the IL-12 clone (IL/14) (Figure 4.2B). Finally, a portion of the MICA/IL-12 fusion protein including the linker segment was detected only in the fusion gene clones M-IL/4 and M-IL/19. This data indicates that the clones have in fact taken up the exogenous DNA and transcription of the genes of interest can be detected.

It is important to demonstrate that the fusion protein is produced and being released from the cell into the tumor microenvironment where it can interact with killer cells. To test this, supernatants were collected from all the clones together with A549 cells and screened using ELISA. As Figure 4.3 shows, the engineered proteins are produced by the respective clones and released from the cells into the supernatants. Clone M-IL/4 produces a relatively high level of fusion protein MICA/IL-12, but it is not detectable in the supernatant collected from M-IL/19 (Figure 4.3A). Because there was a

clear band detected after RT-PCR indicating that M-IL/19 does contain the fusion gene, it is possible that the protein amount produced by this clone is beneath the detection threshold of the methods used. Both clones M-IL/4 and IL/14 produce significant levels of IL-12. It should also be noted that the amount of IL-12 detected in the clone M-IL/4 supernatant is similar to the amount of IL-12 detected in the clone IL/14 supernatant. This indicates that clone IL/14 is a good control for this experiment (Figure 4.3B). As expected, significant levels of MICA were detected in the supernatants from clones M-IL/4 and M/19. Since there is not a significant difference between the MICA levels of clone M-IL/4 and M/19, clone M/19 is a good control for this experiment (Figure 4.3C).

To determine if fusion protein MICA/IL-12 is functional and has enhanced ability to activate killer cells, a human natural killer cell line, NK92, was co-cultured with A549 cells and clones M-IL/4, M-IL/19, IL/14, and M/19 for 48 hours at a ratio of 10:1. The amount of IFN-γ produced by the NK92 cells was evaluated with ELISA to determine the level of NK cell activation. The NK92 cells co-cultured with clones M-IL/4, IL/14, and M/19 showed significantly higher IFN-γ production than NK92 cells co-cultured with A549 parental cells. Clone IL/14 was also able to increase IFN-γ production significantly compared to NK92 cells that were not cultured with any A549 cells. M-IL/19 was not able to significantly increase IFN-γ production compared to NK92 cells alone (Figure 4.4A). M-IL/4 stimulated production of similar levels of IFN-γ as clone IL/14, which is expected because they both have similar IL-12 expression levels as shown in the IL-12 ELISA (Figure 4.3B). It is interesting that NK92 cells co-cultured with A549 parental cells produced much less IFN-γ when compared with NK92 cells that were not co-

cultured with any cells. This is possibly due to either the production of or the shedding of inhibitory ligands from A549 tumor cells into the supernatant, a phenomenon commonly seen in tumors, which can reduce the activation of the NK92 cells by engaging the inhibitory receptors, such as NKG2A/B, KIR2DL4 and ILT-2.[12,104–107] The results not only confirm the notion that the activation of NK cells and other killer cells depends on the balance of inhibitory receptors and activating receptors, but also demonstrate that the fusion protein MICA/IL-12 can change the balance towards activation.

To determine if the fusion protein could enhance NK92 cytotoxicity toward the tumor cells, the number of A549 cells remaining after the co-culture was measured. There were significantly fewer cells of clones M-IL/4, M-IL/19, and IL/14 when compared to the A549 parental cells or clone M/19 cells (Figure 4.4B). Although the cytotoxicities among clones M-IL/4, M-IL/19, and IL/14 are not statistically different, clone M-IL/4 shows a slightly higher activity. This indicates that clone M-IL/19 does produce some MICA/IL-12 even though it was undetectable using ELISA (Figure 4.3C). This suggests that when compared to a larger amount of IL-12, even a small amount of the fusion protein can produce similar cytotoxic results. This is also interesting because clone M-IL/19 was not able to increase IFN-γ production of the NK92 cells whereas M-IL/4 and IL/14 were (Figure 4.4A). This suggests that the addition of the MICA portion of the protein increases the cytotoxic activity of the NK92 cells.

One of the current hurdles for gene therapy is the low efficiency of gene delivery.[108] With this in mind, it is important to determine if MICA/IL-12 can also

activate NK92 cells to kill A549 cells that do not express the protein. NK92 cells that were primed for 48 hours with the supernatants from A549 cells and clones of M-IL/4, M-IL/19, IL/14, and M/19 were co-cultured with parental A549 cells at a ratio of 10:1 and incubated 24 hours. After the removal of the suspension NK92 cells, the remaining tumor cells were measured using an MTS cell proliferation assay. All NK92 cells, whether primed or not, were able to significantly reduce the A549 cell number compared to the control without NK92 cells. This is not a surprise as these cells are highly cytolytic. Both clones M-IL/4 and M-IL/19 primed NK92 cells were able to kill significantly more A549 cells than either A549, IL-4, or M/19 primed NK92 cells. This suggests that priming with MICA/IL-12 is more effective than priming with either IL-12 or MICA alone, even when the amount of MICA/IL-12 is small as seen in clone M-IL/19. Interestingly, the unprimed NK92 cells killed significantly more A549 cells than all other NK92 cells except the NK92 cells primed with MICA/IL-12 (Figure 4.4C). This suggests that MICA/IL-12 expression, even in small amounts, was able to overcome the inhibitory signals from A549 cells during NK92 priming.

Although the NK92 cell line is a good model for human NK cells, it does not give a complete picture of the *in vivo* effects of fusion protein MICA/IL-12. To better understand how MICA/IL-12 will behave in a patient, further experiments were conducted using human PBMCs isolated from leukapheresis products. Supernatants collected from clones M-IL/4, M-IL/19, IL/14, or M/19 were used to treat PBMCs for 10 days and then a relative cell number was determined. Only supernatant from clone M-IL/4, that expresses the highest level of the fusion protein, is able to significantly increase

PBMC cell number (Figure 4.5A), indicating that MICA/IL-12 is able to either increase proliferation and/or increase sustainability of these cells.

An IFN-γ ELISA was also used to detect NK and other killer cell activation in PBMC culture. As Figure 4.5B shows, supernatant from clone M-IL/4 stimulates PBMCs to produce the most IFN-γ, although supernatant from clone M/19 also stimulates PBMCs to produce relatively high levels of IFN-γ. M-IL/4 induced a significantly higher production of IFN-γ when compared to M-IL/19 and IL/14. (Figure 4.5C)

An interesting observation from this study is that the IFN-γ production by unprimed PBMCs is significantly higher than those primed with any clones. This, again, is possibly due to either the production of or the shedding of inhibitory ligands into the supernatant by the tumor cells.[12,104–106] This effect is more pronounced with the PBMCs compared to NK92 cells (Figure 4.4A) as the IFN-γ production by unprimed PBMCs is higher than that of PBMCs primed with supernatants from any of the tumor cell clones (Figure 4.5B), while IFN-γ production by NK92 cells primed with clones M-IL/4, IL/14, or M/19 is higher than that of the unprimed NK92 cells. This is probably due to the PBMCs' larger repertoire of inhibitory receptors. Although the fusion protein MICA/IL-12 did not completely recover the inhibitory effects as it does in NK92 culture, it is much more effective than IL-12 or MICA alone.

Another interesting observation seen when comparing the NK92 and the PBMC results is the effectiveness of the IL/14 and M/19 clones. In the experiments conducted using the NK92 cells, the clone that produced only IL-12 (IL/14) performed better than

the clone that produced only MICA (M/19). The IL-12 was able to initiate production of more IFN-γ by NK92 cells and resulted in more cell death during the co-culture compared to M/19 (Figures 4.4A and 4.4B). However, in the PBMC experiments, clone M/19 initiated the production of more IFN-γ by NK92 cells than was initiated by clone IL/14 (Figure 4.5B). This suggests that the IL-12 may be a more effective activator of NK cells, but the MICA can activate other killer cell types within the heterogeneous PBMC population. The fusion protein, however, consistently outperforms both individual components when tested separately.

In conclusion, a fusion protein containing the extracellular domain of the NKG2D ligand MICA and IL-12 was successfully created and can be stably expressed by A549 tumor cells. The fusion protein can increase the production of IFN-γ by NK92 cells and also increase the cytolytic activity of these cells toward A549 cells expressing MICA/IL-12. The bystander effect was also demonstrated when MICA/IL-12 primed NK92 cells were able to kill parental A549 cells significantly more than NK92 cells primed with either IL-12 or MICA alone. This result was also an indication that the activation by MICA/Il-12 was able to overcome inhibitory signals by the A549 cells. Preliminary studies also indicate that MICA/IL-12 can increase proliferation and/or sustainability of isolated human PBMCs. The data also suggests that MICA/IL-12 is effective at augmenting the IFN-γ production of cells cultured with soluble inhibitory factors produced by A549 cells. Although the study is preliminary, the data, along with the data from the previous mouse studies suggest that the MICA/IL-12 bifunctional fusion protein is an effective activator of killer cells for cancer treatment.

Materials and Methods

Cells

The human lung carcinoma cell line A549 (ATCC no. CRM-CCL-185) was cultured in F12K medium containing 10% fetal bovine serum (FBS) and 100 μg/ml gentamicin at 37°C with 5% CO_2. Human natural killer cell line (CRL-2407) NoGFP-CD16.NK92 (PTA-6967) and the freshly isolated human PBMCs were cultured in α-MEM with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.2 mM inositol, 0.1 mM β2-mercaptoethanol, 0.02 mM folic acid, 200 U/ml recombinant IL-2, 12.5% horse serum, 12.5% FBS, and 100 μg/ml gentamicin at 37°C with 5% CO_2. The blood samples were procured following a GHS IRB approved protocol (CC/ORI 07-02) and the patients consented that their blood materials would be used for research purposes.

Construction of fusion gene and control gene Vectors

pcDNA3.1(+)MICA/IL-12 The MICA extracellular domain sequence was amplified from pCMV-SPORT6-MICA (Open Biosystems, Lafayette, CO, USA) using the 5' primer CCCAAGCTTGAGAGGGTGGCGACGTCGGGG, the 3' primer CGGGATCCCTGCC AATGACTCTGAAGCACC, and Phusion High-Fidelity DNA Polymerase. The 5' primer contains a restriction cut site for HindIII and the 3' primer contains the restriction cut site for BamHI. The PCR fragment was excised and gel purified using a gel purification kit. Double enzyme digestion using HindIII and BamHI was performed on the purified fragment. The plasmid pcDNA3.1(+)MULT1E/mIL-12, created earlier in our lab,[100] was also digested with HindIII and BamHI to remove the MULT1E and

allowing for the insertion of MICA directly upstream of a (GGGS)3 linker and IL-12 in frame. The linearized plasmid was excised and gel purified using a gel purification kit. The enzyme digested MICA PCR fragment was then ligated to the vector creating the new plasmid pcDNA3.1(+)MICA/IL-12 (Figure 4.1). The sequence was confirmed using DNA sequencing.

pcDNA3.1(+)IL-12 Vector To make a control IL-12 vector, the IL-12 sequence was amplified from pORF-mIL-12 using the 5' primer CCAAGCTTCCATGGGTCAATCAC GCTACCTCC with a HindIII cut site and the 3' primer CCTCGAGCTAGGATCGGAC CCTGCAGGG with a XhoI restriction cut site using Phusion High-Fidelity DNA Polymerase. The fragment was excised and gel purified using a gel purification kit. Double enzyme digestion was performed on the purified fragment using HindIII and XhoI. pcDNA3.1(+)Neo was digested with HindIII and XhoI allowing for the insertion of IL-12. The fragment was excised and gel purified using a gel purification kit. The IL-12 sequence was then ligated to pcDNA3.1(+)Neo creating the new plasmid pcDNA3.1(+)IL-12. The sequence was confirmed using DNA sequencing.

pcDNA3.1(+) MICA Vector To make a control MICA vector encoding a secretable form of the extracellular domain of MICA, the extracellular MICA sequence was amplified from pcDNA3.1(+)MICA/IL-12. The 5' primer was the same used for the construction of pcDNA3.1(+)MICA/IL-12 and the 3' primer (CCTCGAGCTACTGCCAATGACT CT) included a stop codon and the restriction site for XhoI. PCR was performed to amplify MICA using Phusion High-Fidelity DNA Polymerase. The fragment was

excised and gel purified using a gel purification kit. Double enzyme digestion was performed on the purified fragment using HindIII and XhoI. pcDNA3.1(+)Neo was digested with HindIII and XhoI allowing for the insertion of MICA. The fragment was excised and gel purified using a gel purification kit. The MICA sequence was then ligated to the pcDNA3.1(+)Neo creating the new plasmid pcDNA3.1(+)MICA. The sequence was confirmed using DNA sequencing.

Cell transfection

A549 cells were transfected with either pcDNA3.1(+)MICA/IL-12, pcDNA3.1(+)IL-12, or pcDNA3.1(+)MICA using Lipofectamine as directed by the manufacturer. To obtain stable clones, the transfected A549 cells were cultured in medium containing 250 μg/ml G418. Drug-resistant clones were collected and subcultured in the presence of the appropriate drug.

Proliferation Assay

To determine if the selected clones grow at the same rate as the untransfected parental A549 cells, cells were counted every day for 5 days. On day one, $0.05x10^6$ cells per well were plated on 96 well plates in triplicates. At each time point, Promega's CellTiter 96 AQ_{ueous} nonRadioactive cell proliferation assay was used following the manufacturer's instructions to determine the relative cell count. Clones were compared using a two-way ANOVA with Tukey's post-test.

RT-PCR

Total RNA was extracted from each clone using an RNeasy Plus Mini Kit following the manufacturer's directions. RT-PCR was run using 1ug of the total RNA using the Phusion RT-PCR kit following the manufacturer's directions. To amplify a 177 bp segment from MICA the 5' primer CCTTGGCCATGAACGTCAGG and the 3' primer CCTCTGAGGCCTCGCTGCG were used. To amplify an 805 bp sequence of IL-12 the 5' primer GGGTGATGGGCTATCTGAGC and the 3' primer AACTTGAGGGA GAAGTAGGAATGG were used. To amplify a 1.2 kb portion of the MICA/IL-12 fusion gene containing 446 bp of MICA, the (GGGS)3 linker, and 708 bp of IL-12 the 5' primer CCTTGGCCATGAACGTCAGG and the 3' primer GGGAGTCCAGTCCAC CTCTA were used. All reactions included the GAPDH housekeeping gene using the 5' primer ATGACATCAAGAAGGTGGTG and the 3' primer CATACCAGGAAATGAGCTTG.

Detection of Secreted Protein

Cells of different clones were plated at $2x10^6$ cells in 6 well plates with normal growth media on day one. On day two, the media was removed, the cells were rinsed with PBS, and serum free F12K was added. On day three, the serum free RPMI was removed and centrifuged at 1000g for 15 min. The supernatant was then tested using an appropriate immunofluorescence or ELISA protocol.

To detect the presence of MICA/IL-12, 96 well plates were coated with 2 μg/ml rhNKG2D/Fc chimera in carbonate coating buffer and incubated overnight at 4^oC. The plates were rinsed with PBS and blocked with 10% FBS for 1.5 hours. After washing

three times with PBS, 100µl of the cell supernatant was added to the plate in triplicates. The samples were incubated at room temperature for 3 hours. After removing the samples and washing the plates three times with PBS, 1 µg/ml PE rat anti-mouse IL-12 (p40/p70) in blocking solution was added to the plates. The plates were then incubated for 1.5 hours at room temperature. After washing five times with PBS, the plates were read with a BioTek Synergy H1 hybrid reader. Clones were compared using a one-way ANOVA with Tukey's post-test. Corrected RFU is calculated by subtracting background RFU from each value.

To detect the presence of secreted IL-12 from the cells, a mIL-12 p70 Ready-SET-Go! ELISA kit was used following the manufacturer's directions. Plates were read with a BioTek Eon microplate reader. Clones were compared using a one-way ANOVA with Tukey's post-test.

To detect the presence of MICA, 96 well plates were coated with 2 µg/ml rhNKG2D/Fc chimera in carbonate coating buffer and incubated overnight at 4^{o}C. The plates were rinsed with PBS and blocked with 10% FBS for 1.5 hours. After washing three times with PBS, 100µl of the cell supernatant was added to the plate in triplicates. The samples were incubated at room temperature for 3 hours. After removing the samples and washing the plates three times with PBS, 10 µl anti-hMICA phycoerythrin conjugated in 90 µl blocking solution was added to the plates. The plates were then incubated for 1.5 hours at room temperature. After washing five times with PBS, the plates were read with a BioTek Synergy H1 hybrid reader. Clones were compared using

a one-way ANOVA with Tukey's post-test. Corrected RFU is calculated by subtracting background RFU from each value.

NK92 and A549 Co-culture

NK92 cells were plated with A549 cells or clones expressing the fusion protein, IL-12, or MICA on a 96 well plate at a ratio of 10:1 in 250 µl NK media and incubated for 48 hours. After the incubation, 100 µl of media was removed and analyzed for the presence of IFN-γ using Thermo's human IFN-γ ELISA reagent kit following the manufacturer's directions. The rest of the media was removed from the cells, the NK suspension cells were carefully rinsed off, and 100 µl fresh NK media was added. The remaining tumor cell number was determined using Promega's CellTiter 96 AQ_{ueous} nonRadioactive cell proliferation assay following the manufacturer's instructions. Clones were compared using a one-way ANOVA with Tukey's post-test.

Primed NK92 Cytotoxicity

The A549 clones were plated on a 6 well plate in 5ml NK media at a density of 0.5×10^6 cells per well and incubated for 48 hrs. After the incubation, the supernatant was collected from these cells and used to plate 4×10^5 NK92 cells on a 6 well plate. After a 48 hr priming period, these NK92 cells were removed and plated on a 96 well plate at a density of 5×10^4 cells per well with 0.5×10^4 parental A549 cells and incubated for 24 hrs. After the incubation, the media was removed from the cells and the wells were carefully washed four times to remove the non-adherant NK92 cells. 100 µl fresh NK media was added. Cell proliferation was determined using Promega's CellTiter

96 AQ_{ueous} nonRadioactive cell proliferation assay following the manufacturer's instructions. Clones were compared using a one-way ANOVA with Tukey's post-test. Corrected OD is calculated by subtracting background OD from each value

Preparation of human PBMCs

PBMCs were isolated from leukapheresis product using the Ficoll-Paque method. Briefly, the blood product was mixed 1:4 with PBS and loaded on top of 30 ml lymphocyte separation media and centrifuged for 15 min. The PBMC layer was then removed and rinsed with PBS. ACK lysing buffer was then used to lyse any remaining RBCs. After a final PBS rinse, cells were counted and used.

Supernatant Transfer to PBMC

The A549 clones were plated on a 6 well plate in 1.5ml PBMC media at a density of 0.6×10^6 cells per well and incubated for 48 hrs. After the incubation, the supernatant was collected from these cells and used to plate 2×10^5 freshly isolated PBMCs on a 96 well plate and the cells were incubated for 10 days. After the incubation, 100 µl of media was removed and analyzed for the presence of IFN-γ using Thermo's human IFN-γ ELISA reagent kit following the manufacturer's directions. The rest of the media was removed from the cells and 100 µl fresh PBMC media was added. Cell proliferation was determined using Promega's CellTiter 96 AQ_{ueous} nonRadioactive cell proliferation assay following the manufacturer's instructions. Clones were compared using a one-way ANOVA with Tukey's post-test.

Statistics

GraphPad software was used to plot graphs and run statistics. The significance was represented as $^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$.

Acknowledgements

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CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

One of the characteristics of advanced tumors is the evasion of the immune system. There are multiple methods that tumor cells employ to achieve this including reducing the expression of activating ligands on the cell surface and a shift in the tumor microenvironment toward pro-tumor cytokines. The purpose of this research is to develop a novel bifunctional fusion protein that will target these two deficiencies in the tumor microenvironment and activate killer cells that are already present.

The mouse form of a fusion gene encoding the extracellular domain of the NKG2D ligand mouse UL-16-binding protein-like transcript 1 (MULT1E) and mouse IL-12 (mIL-12) was created with modern DNA cloning technology: pcDNA3.1(+)MULT1E/mIL-12. Mouse tumor cells were transfected with the fusion gene construct. When expressed by the engineered tumor cells, the fusion protein MULT1E/mIL-12 indeed activated NK cells *in vitro* as assayed by increased production of IFN-γ and cytotoxicity. Tumor cells that produce the fusion protein were then intravenously injected into female C57BL/6J mice to determine the anti-tumor activity *in vivo*. MULT1E/mIL-12 significantly reduced the number of tumor nodules found on the lungs after 4 weeks with the highest producing clone (M-IL/4) having no tumor nodules at all. MULT1E/mIL-12 also performed better than both a clone that only produced the MULT1E portion of the protein (MULT/6) and a clone that produced only the mIL-12

portion of the protein (IL/3) in both *in vitro* and *in vivo* studies.

To expand the concept of developing a novel bifunctional fusion protein for enhanced killer cell activation to human killer cells, the mouse NKG2D ligand MULT1 was replaced with the extracellular domain of a human NKG2D ligand MHC class I polypeptide-related sequence A (MICA). The fusion protein, when expressed by engineered tumor cells, indeed activated a human NK cell line, NK92, as measured by an increase in IFN-γ production and an increase in cytotoxicity of tumor cells. In addition, MICA/IL-12 was able to prime the NK92 cells and increase their cytotoxicity toward tumor cells that did not produce the protein. This is important because it demonstrates that even if gene delivery is not highly efficient, MICA/IL-12 can increase killing of non-transformed cells. This experiment also showed that the fusion protein was able to counteract the inhibitory factors found in the tumor cell media and returns the killing ability to uninhibited levels.

Although the NK92 cell line is a good model for human NK cells, it does not give a complete picture of the *in vivo* effects of the fusion protein MICA/IL-12. To test this, human peripheral blood mononuclear cells (PBMCs) collected from leukapheresis products was tested. Only media containing the fusion protein was able to increase the proliferation of human PBMCs while media containing just IL-12 or just MICA could not. MICA/IL-12 was also able to augment PBMC production of IFN-γ.

This study suggests that a bifunctional fusion protein containing an NKG2D ligand and IL-12 represents an effective activator of killer cells for cancer treatment,

however there are still questions that need to be answered. First, what is the primary mechanism of action *in vivo*? This question can be addressed by determining if NK cells are in fact activated *in vivo*. To determine this, cells from different clones can be inoculated in to NK cell-deficient beige KO mice (C57BL/6J-Lystobg-J/J)[97] and tumor development can be monitored. We speculate that the tumors will grow faster in beige mice when compared with regular C57 BL/6J mice.

Also, in both the mouse and human studies, the NK cells performed differently than the heterogeneous cell populations that contained other killer cells (the *in vivo* mouse model and the PBMCs). This indicates that NK cells are not the only killer cell population that is activated by the fusion protein. This is not surprising because other cell types contain the NKG2D receptor and/or the IL-12 receptor including CD8+ T cells, NKT cells, and CD4+ T cells.[39,62,63] Once activated by IL-12, many of these cell types produce IFN-γ which has many important biological functions including the activation of type I macrophages.[77] Several approaches could be used to identify the effector cell types involved in the anti-tumor response seen in these experiments. The use of KO mice has already been mentioned and may also prove beneficial in elucidating this question. Additionally, lungs could be collected from mice that received the intravenous injection of engineered tumor cells producing the fusion protein and stained to identify the specific cell populations. This could be done either by immunohistochemistry or a more quantitative number could be achieved after tissue disruption and FACS. A similar cell staining and count method could be carried out on the PBMC culture after priming with the fusion protein to determine which cell types are increased after activation.

Furthermore, the activity may be a result of the production of IFN-γ that has been shown to be one of the primary mediators of IL-12 activated anti-tumor activity.[67] To determine if the effects seen were modulated primarily by the fusion protein directly activating killer cells or by the production of IFN-γ, a neutralizing antibody to IFN-γ could be added.

Second, the pulmonary metastasis data cannot differentiate whether the fusion protein prevents implantation of the tumor cells by causing killer cells to destroy them en route or if killer cells destroy the tumor cells post-implantation. To answer this question, a gene delivery vehicle can be used to deliver the fusion gene directly to already established tumors. Adenoviral vectors have been used previously in our lab making them a convenient choice.[59] Adenoviral vectors can be produced that are cancer-specific by including cancer-specific promoters, like the human telomerase (hTERT) promoter that is active in 85-90% of tumor tissues but is inactive in most normal host tissues.[109] We will also investigate the possibility of using nanoparticles to deliver the fusion gene specifically to tumors using tumor-specific surface markers.[110] Either of these mechanisms will achieve the ultimate goal of making this novel fusion protein an effective cancer immuno/gene therapy.

Another concern is the side effects of using this bifunctional protein. We did not observe any side effects in the preliminary *in vivo* mouse study. The ultimate goal is to use this fusion protein as a novel immuno/gene therapy. Once the delivery vehicle is determined, future studies can more broadly analyze the effectiveness and potential side

effects of this treatment. One of the key concerns will be how to make sure the gene is delivered into tumor cells specifically, but not into other healthy cells because if the gene is expressed by healthy cells, the fusion protein may activate NK cells in healthy tissues and cause off-target effects and toxicity. Several methods to prevent this were mentioned previously.

In conclusion, the novel fusion protein consisting of the extracellular domain of an NKG2D ligand and IL-12 is an effective activator of killer cells both *in vitro* and *in vivo*. This was shown in both mouse and human models. Because this novel fusion protein activates killer cells that are part of the innate immune system that does not require the use of tumor specific antigens, this therapy should be easily translatable to many different tumor types. Using gene therapy to deliver the fusion protein directly to the tumor cells will maintain a high level of IL-12 in the tumor microenvironment while providing low systemic IL-12 levels, reducing toxicity.

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